Role of Notch Signaling in Cell Fate Determination of Dental Epithelial Stem/Progenitor Cells

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1. Summary

During embryogenesis, ectodermal stem cells adapt different fates and consequently are able to generate various ectodermal organs like teeth, salivary glands and mammary glands. Mouse incisor is a suitable model to study the regulatory mechanisms of dental epithelial stem cells (DESCs) due to its continuous growth. The aim of this study is to investigate the role of Notch signaling in cell fate determination of dental epithelial stem/progenitor cells. After establishing optimal conditions to expand DESCs from the cervical loop (cl) area of the incisor, we showed that Notch is required for the proliferation and survival of DESCs and it is preventing DESC differentiation. In order to study the role of Notch signaling *in vivo*, we have analyzed K14N1cKO mice. However, we have not observed any major defect in dental epithelium, most probably because Notch1 was not efficiently deleted from all dental epithelial cells, indicating that a more efficient Cre driver mouse line should be used for this purpose. Moreover, by using lineage-tracing assays, we have identified a new population of Notch1-expressing dental epithelial stem cells that strongly contribute to generate enamel-producing ameloblasts in a severely injured postnatal incisor.

2. Zusammenfassung

Ektodermale Stammzellen können während der Embryogenese unterschiedliche Differenzierungswege einschlagen und sind somit in der Lage unterschiedliche Gewebe, wie beispielsweise Zahngewebe, Speichel- oder Brustdrüsengewebe, zu bilden. Aufgrund ihres kontinuierlichen Wachstums sind die Schneidezähne der Maus ein gutes Modell um die Mechanismen der dentalen Stammzellregulation und Differenzierung zu studieren. Das Ziel der vorliegenden Arbeit ist die Untersuchung der Notch-Signalkaskade bei der Differenzierung epithelialer Stammzellen im Zahn. Nach der Etablierung geeigneter Methoden zur Anreicherung dentaler epithelialer Stammzellen (DESC) aus dem cl Gebiet des Schneidezahns der Maus, konnten wir zeigen, das der Notch-Signalweg für die Proliferation und das Überleben der DESC von entscheidender Bedeutung ist und eine weitere Differenzierung der Zellen verhindert. Um den Signalweg in vivo zu untersuchen verwendeten wir das K14N1cKo Mausmodell. Hierbei konnten keine wesentlichen Defekte im Bereich des Zahnepithels nachweisen werden. Dieser Befund erklärt sich am ehesten damit, dass Notch1 nicht vollständig aus den untersuchten Epithelzellen eliminiert wurde. Zukünftige Untersuchungen sollten daher mittels effizienteren Cre Drivern durchgeführt werden. Mittels in vivo Analyse der genetischen Abstammung gelang es uns eine neue Population von Notch-1 positiver DESC zu identifizieren, welche wesentlich an der Bildung von zahnschmelzproduzierenden Ameloblasten im geschädigten postnatalen Schneidezahn der Maus beteiligt ist.

3. Introduction

3.1 Organogenesis

Organogenesis is a series of organized integrated events that transforms an amorphous mass of cells into a complete organ in the developing embryo (Gilbert S., 2000). This continues and organised events result from reciprocal interaction between cells and their microenvironments. Inductive interactions imply the presence of an inductor that signals a given tissue that for being competent can reply to such signal. In addition, these signals are usually reciprocal, meaning that the inductor turns into an induced tissue. Inductive interaction may take place in variety ways in different tissues. Most common interactions are the epithelial-mesenchymal interactions. Epithelia are sheets of connected cells that can originate from any germ layer (ectoderm, endoderm or mesoderm) whereas mesenchyme refers to loosely associated cells that arise from either mesoderm or ectoderm-derived neural crest (Thesleff et al., 1995).

There are two main mechanisms for the transmission of signals between the inductor tissue and the one that responds, and they are called paracrine and juxtracrine signalings. In paracrine signaling, molecules diffuse and stimulate nearby cells and juxtacrine signaling involves cell to cell contact (Salder et al., 2000; Ribatti & Santoiemma, 2014).

3.2 Ectodermal organs

Teeth, salivary glands, mammary glands and skin appendages, are all organs of ectodermal origin that despite the diversity in form and function share several common features (Jiménez-Rojo et al., 2012). All ectodermal appendages develop through continuous and reciprocal interactions between the ectoderm-derived epithelium and the underlying mesenchyme. Additionally, they share regulatory mechanisms and present similar developmental stages. During the initiation stage, an epithelial thickening forms the ectodermal placode followed by the condensation of the underlying mesenchymal cells. Then, the epithelium grows and evaginates (in the case of feathers) or invaginates into the

mesenchyme (in the rest of ectodermal organs), forming an epithelial bud. Then the ectodermal organ enters the next developmental stage, morphogenesis, when the bud grows, and either folds or branches acquiring the final shape and size of the organ (Fig. 1) (Pispa & Thesleff 2003; Jiménez-Rojo et al., 2012). Finally, during differentiation stage, cells specialize and form the specific structures of the given organ.

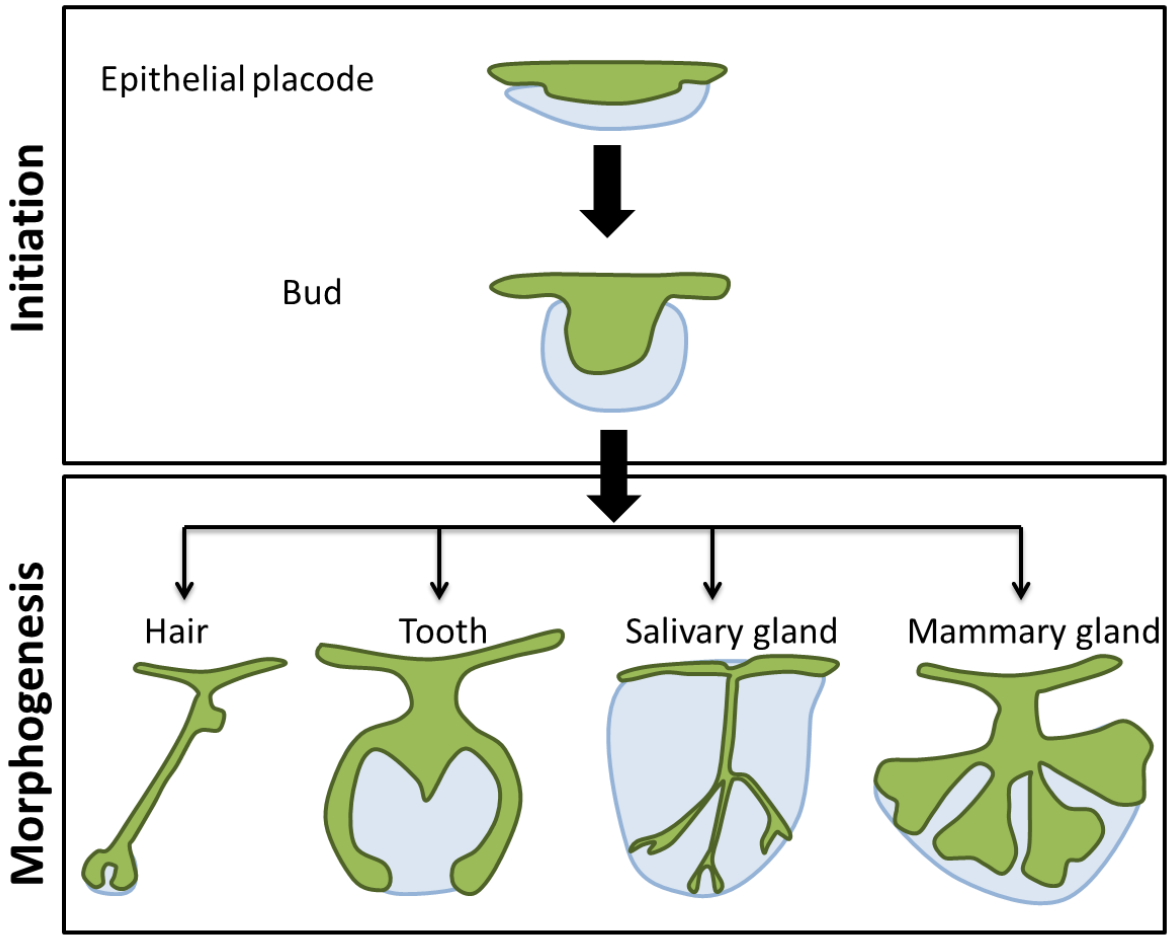


Figure 1: Developmental stages of ectodermal organs. Ectodermal organs develop though a continuous crosstalk between the epithelium (green colour) and the mesenchyme (blue colour). First, the epithelium thickens in order to form the placode, and generally, the bud grows further into the mesenchyme resulting in the acquisition of the final shape of the organ.

3.2.1 Epithelial stem cells during homeostasis and regeneration of ectodermal organs

Although the main morphogenetic processes of ectodermal organs occur during embryogenesis, most of them undergo their terminal differentiation and become functional at postnatal stages. Tissue homeostasis throughout life includes the regular elimination and replacement of differentiated cells preserving the form and function of developed tissues and organs by cell turnover (Pellettieri & Sánchez Alvarado 2007; Watt & Driskell 2010). Ectoderm-derived skin and oral cavity, as well as the highly specialized appendages that arise from them, are in close contact with the environment. Consequently, these organs are exposed to a great risk of being damaged and it becomes extremely important that they retain regeneration capacity throughout the organisms' life. Tissue homeostasis and wound repair during postnatal stages are ensured by adult stem cells, located within a specialized microenvironment, which is called niche (Blanpain & Fuchs 2014). To date, different methods have been used to identify and characterize adult stem cells from ectodermal organs. For instance, fluorescence activated sorting (FACS) has been used to isolate different cell populations residing in a given tissue in order to further assess their stemness by using techniques such as spheres and colonies-forming assays (Barrandon & Green 1987; Blanpain et al., 2004; Lombaert et al., 2008; Dontu et al., 2003) or transplantation assays (Blanpain et al., 2004; Lombaert et al., 2008; Miletich & Sharpe 2016). In addition, lineage-tracing experiment using inducible Cre-lox technology have been used in order to identify adult stem cells under more physiological conditions in a number of ectodermal organs (Van Keymeulen et al., 2011; Juuri et al., 2012; Biehs et al., 2014; Seidel et al., 2010; Levy et al., 2007).

Thus, it has been shown that epithelial stem cells from postnatal ectodermal organs have the capacity to give rise to all the epithelial-specific lineages during normal physiological conditions. Interestingly, those stem cells can present higher plasticity and contribute to the formation of cell lineages from different organs upon injury repair processes (Levy et al., 2007; Blanpain & Fuchs 2014; Van Keymeulen et al., 2011; Levy et al., 2005).

3.3 Tooth as a model for the study of ectodermal organs

Teeth are ectodermal organs derived from oral ectoderm and neural crest-derived mesenchyme. Tooth has served as a great tool to better understand the nature of epithelial-mesenchymal interactions through classical tissue recombination experiments (Sengel 1990; Kratochwil 1969). In addition, during the last decades, tooth has been a valuable model for the study of the molecular mechanisms responsible for such interactions (Mina and Kollar, 1987) (Mitsiadis et al., 2003; Bartlett 2013).

3.3.1 Tooth

Teeth are highly specialized organs located in the oral cavity that consist of four different tissues, which are enamel, dentin, dental pulp and cementum. The part of a tooth exposed to the oral cavity is known as the tooth crown, and the part below the tooth crown is known as the tooth root. The tooth crown is covered by enamel, which is a hard, highly mineralised tissue that protects it from erosion (Mitsiadis & Graf 2009). Dentin, a hard, but less mineralised tissue, can be found in both crown and roots. Another part of the tooth, the dental pulp is covered by dentin and is highly innervated and vascularized. Cementum is the tissue that covers the surface of the tooth root and it connects the alveolar bone with the tooth by the periodontal ligament (Nanci, 2012) (Fig. 2).

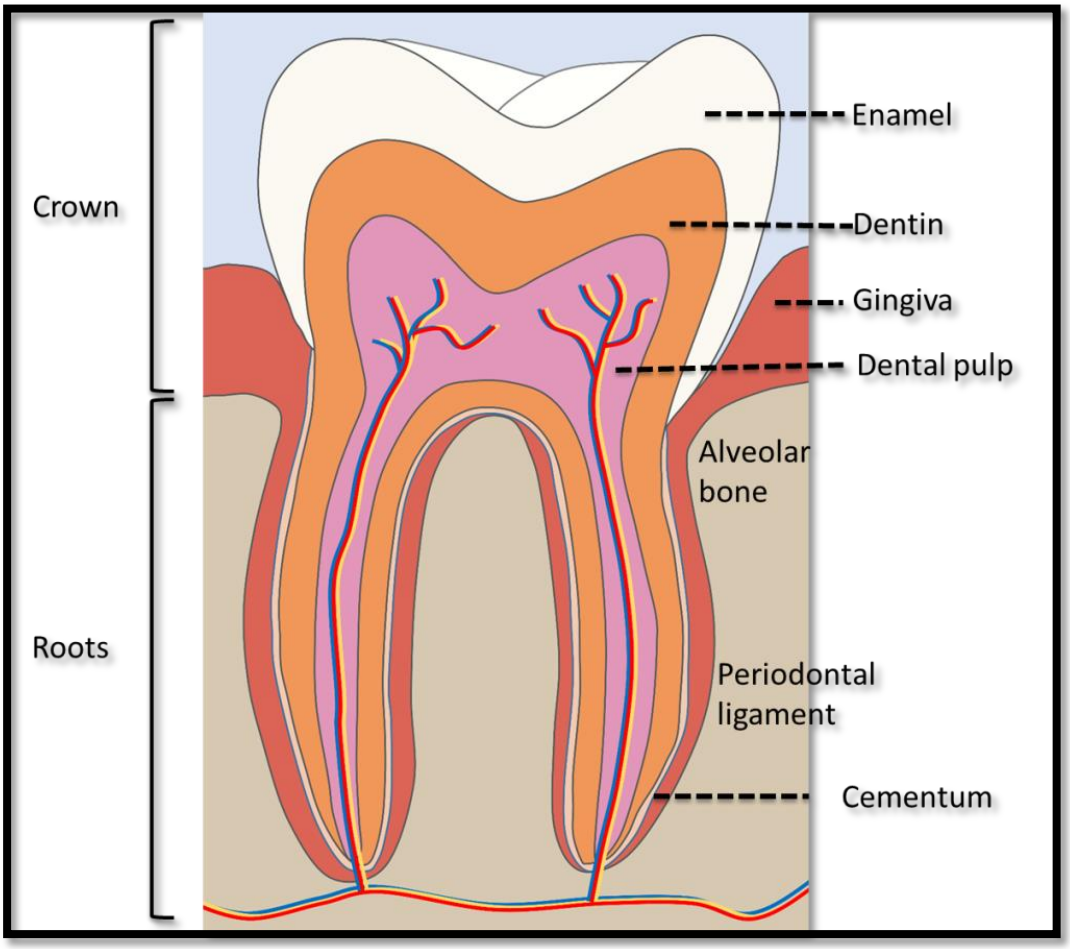


Figure 2: Schematic representation of the anatomy of a human tooth. The tooth is composed of enamel, dentin, cementum, and the dental pulp. The tooth is attached to the alveolar bone of the jaw through the periodontal ligament that binds root cementum to the surrounding bone. Modified figure (Mitsiadis et al., 2012).

3.3.2 Tooth development

Tooth development is regulated by continuous epithelial-mesenchymal interactions. Initially, the first visible sign of odontogenesis is the thickening of the oral epithelium on embryonic day 10.5 (E10.5), which then forms the dental placode. The epithelium grows and invaginates into the mesenchyme and thus the tooth bud is formed at E12.5-E13.5. As buds are growing, at E14.5-E15.5 they acquire a cap shape. At this stage, the epithelium becomes more specialized and the enamel organ is formed. The enamel organ is composed of four distinct epithelial cell populations: inner enamel epithelium (iee), stratum intermedium (si), stellate reticulum (sr) and outer enamel epithelium (oee). During the cap stage, a signaling center

named the primary enamel knot appears. This is composed of a group of epithelial cells that induce the growth of the dental epithelium and afterwards die by apoptosis. At E16.5, the tooth reaches the bell stage and the secondary enamel knots (a cell locus in the enamel layer) regulate the final shape of the tooth and the morphology of the tooth cusps. During the late bell stage (E18.5), dental pulp cells start to differentiate into odontoblasts and cells from the inner enamel epithelium in contact with the newly formed odontoblasts differentiate into ameloblasts (Fig. 3). The differentiation stages of the epithelial cells into functional ameloblasts can be divided into three stages: i) preameloblasts stage, ii) secretory ameloblasts stage and iii) mature ameloblasts stage. In the preameloblast stage, the iee differentiate into preameloblasts. At this stage, preameloblasts extend cytoplasmic projections through the basement membrane. Their cytoplasm contains organelles that are needed for the synthesis of the enamel protein. The initial layer of the enamel is formed but does not contain any rods. During the secretory ameloblasts stage, preameloblasts differentiate into secretory ameloblasts. These ameloblasts are highly polarized and form conical projections known as Tomes' processes in their apical surface. At this stage, the ameloblasts secrete enamel proteins (such as Amelogenin, Ameloblastin and Enamelin) into the enamel matrix and The first enamel crystals (ribbons) form and as the enamel move away from the dentin surface the ribbons will eventually form the main enamel structural unit called enamel rod (or prism). When the enamel layer reaches its full thickness, ameloblasts become shorter and wider. These mature ameloblasts actively secrete enzymes (such as Kallikrein-related peptisade-4) that will digest the previously secreted enamel proteins that served as scaffolds for the deposition of minerals. Consequently, during this last stage, water and most of the organic matrix is removed from enamel leaving free space for the deposition of hydroxyapatite crystals that confer to the enamel its hardness, making it the hardest tissue of the body (Bartlett 2013; Nanci, 2012)

After crown development, both the inner and the outer dental epithelia form the Hertwig's epithelial root sheath. Hertwig's epithelial root sheath-mesenchyme interactions lead to root formation. Generally, most of these stages occur during the embryonic age but root development and tooth eruption take place at postnatal stages. After tooth eruption, most of the dental epithelium, especially the cells forming the enamel organ, disappears by apoptosis (Mitsiadis & Graf 2009; Jernvall et al., 1994)

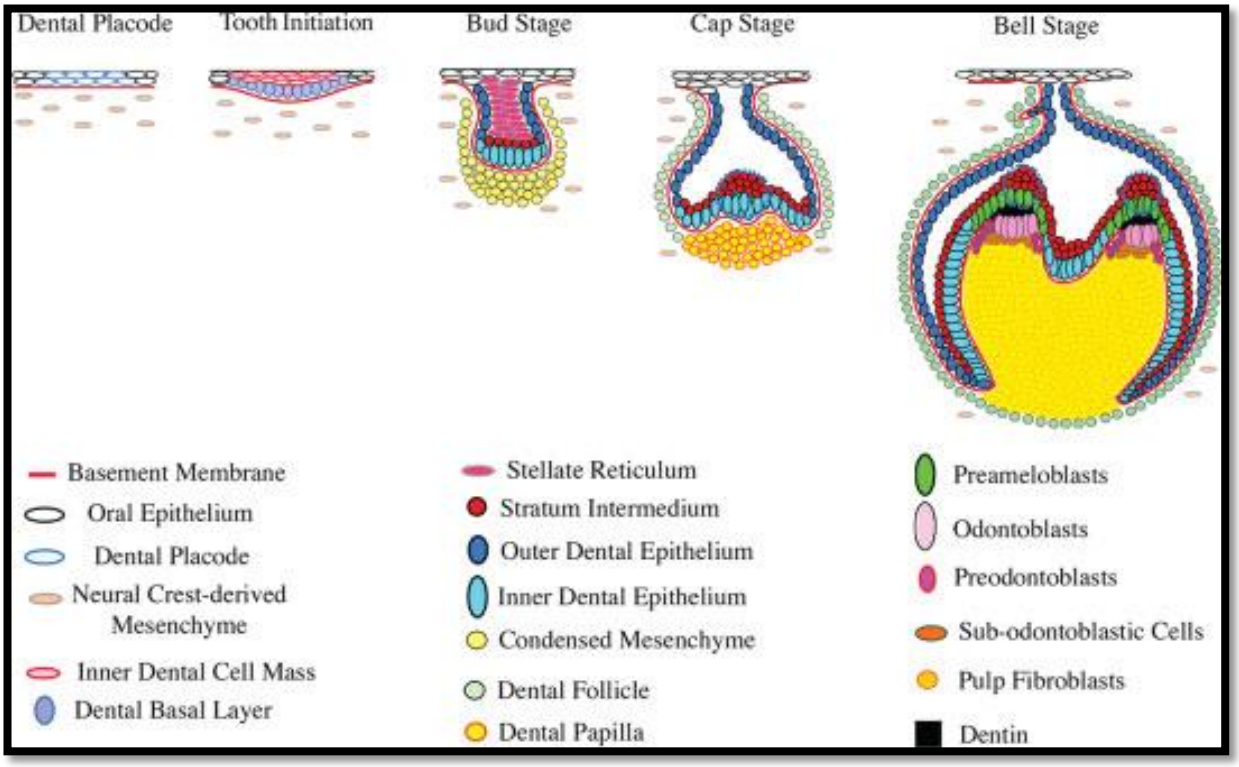


Figure 3: Schematic representation of the main stages of tooth development. Different colours and shapes show the diverse cell fates during tooth morphogenesis. Mitsiadis and Graf., 2009.

3.3.3 Mouse incisor as a model for the study of dental epithelial stem cells

The mouse has in each hemi-mandible three molars and one incisor. Mouse molars have similar anatomical and developmental features to human teeth (Bluteau et al., 2008) (Fig. 4A). However, the mouse incisor has characteristic anatomical features such as an elongated cylindrical shape with a lingual side comparable to roots of the molars and a labial side

equivalent to the crown of the molar including enamel formation. More importantly, the incisor is a continuously growing organ that contains stem/progenitor cells niches in its posterior part (Harada et al., 2002). Epithelial stem/progenitor cells located in an epithelial structure known as cervical loop (cl) are continuously giving rise to new enamel-producing ameloblasts (Jimenez-Rojo et al., 2012b). Epithelial stem cells located in the cl area generate first transit amplifying (TA) cells that are more committed progenitors that will give rise to the four layers of dental epithelium: iee that eventually differentiates into enamel-secreting ameloblasts (Fig. 4C-E), si that is composed by cuboidal cells lining the iee/ameloblast layer, stellate reticulum formed by stellate-shaped cells and oee (Mitsiadis & Graf 2009; Mitsiadis et al., 2011; Catón et al., 2011).

There is a differentiation gradient in the mouse incisor epithelium that goes from the cl where epithelial stem/progenitor cells are located towards the terminally differentiated ameloblasts in the anterior part where the incisor erupts into the oral cavity. This allows to visualize all the cell lineages composing the dental epithelium hierarchy in one single individual which makes mouse incisor a great model for the study of the establishment of different epithelial lineages.

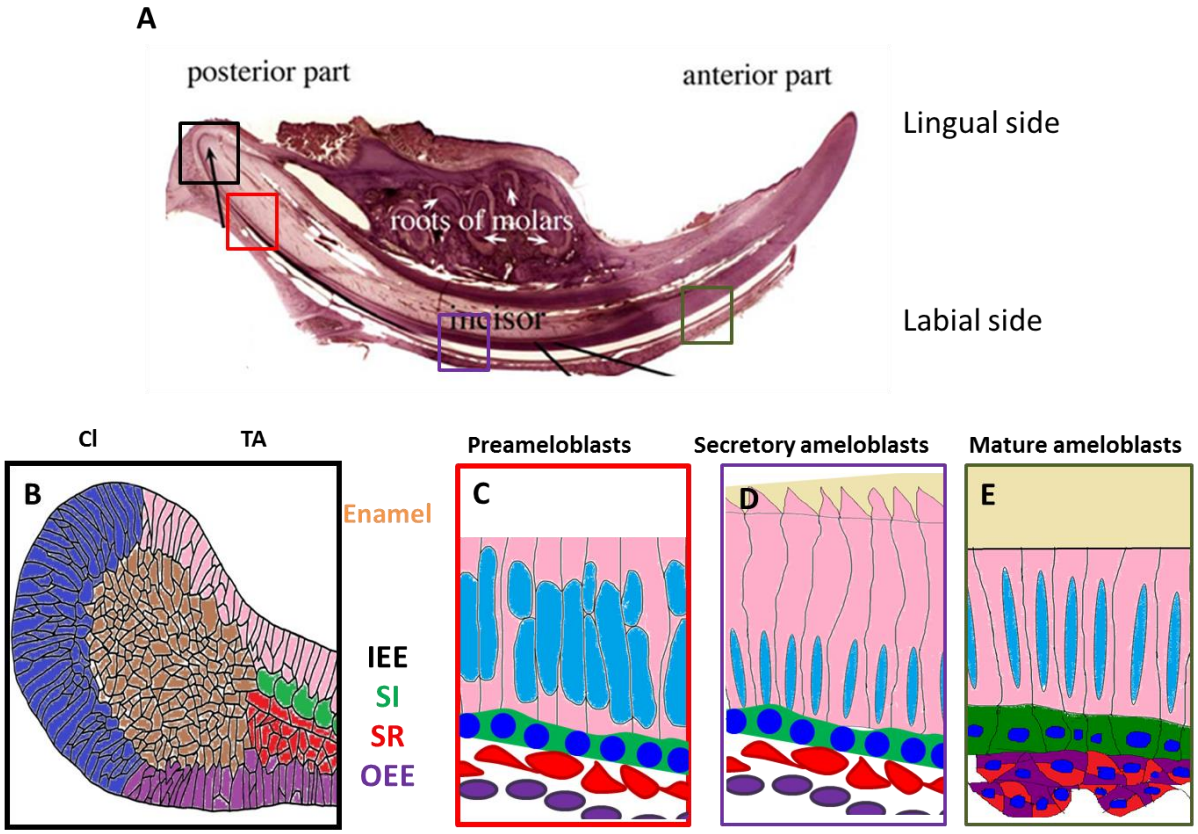


Figure 4: The mouse tooth as an experimental model. (A) Histological overview of the mouse incisor. Adapted from (Bluteau et al. 2008). Schematic representation shows the differentiation stages of the epithelial cells into functional ameloblasts: cl and TA area (B) preameloblasts (C) secretory ameloblasts (D) and mature ameloblasts (E).

3.2.3 Characterization of epithelial stem cells from the mouse incisor

The first evidence that the labial cl contains stem cells and give rise to the rapidly proliferating TA cells came by using labelling assays (Smith & Warshawsky 1975; Smith CE, 1980). Later on, 5-Bromo-2'-deoxyuridine (BrdU) and [1,1'-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3',3'-tetramethylindocarbocyanine] (DiI) labeling assays revealed that stem cells reside in the cl area (Harada et al., 1999). More recently, genetic labelling with a tetracycline inducible histone 2B-Green Fluorescence Protein (H2BGFP) reporter showed that a population of slowly-dividing epithelial stem cells was present in the cl area (Seidel et al. 2010). In the cl area some general stem cell markers such as Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) (Suomalainen & Thesleff 2010), ATP-binding cassette sub-family G

member 2 (ABCG2), Polycomb complex protein (BMI-1), octamer-binding transcription factor 3/4 (OCT-3/4), and Yes-associated protein (YAP) (Li & Clevers 2010) have been shown to be expressed. In addition, it has been observed that incisor cervical loop contains cells with the ability to grow as spheres (Chavez et al., 2013; Chang et al., 2013; Chang et al., 2013b). More recently, CreERT2-inducible technology has been used to label putative dental epithelial stem cells from mouse incisor and follow their progeny. By using this assay, it was demonstrated that the transcription factor SRY (sex determining region Y)-box 2 (Sox2), Bmi1 and Gli1-expressing cells located in the cervical loop contribute to all epithelial cell lineages of the continuously growing postnatal mouse incisor (Fig. 5) (Juuri et al., 2012; Seidel et al., 2010; Biehs et al., 2014; Zhao et al., 2014). However, stem cell hierarchy and the regulation of fate choices in dental epithelium remain unclear and there is very limited if not inexistent information about the behaviour of stem/progenitor cells during injury situations.

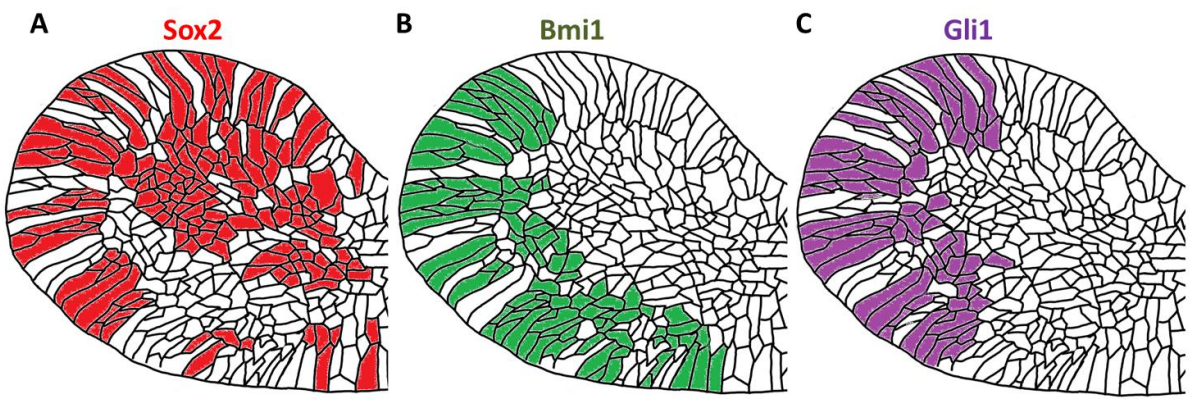


Figure 5: Different populations of stem cells within the cervical loop of mouse incisor.

3.4 Regulation of stem cell fate determination in dental epithelium

In the last 15 years, different studies have shown the presence of epithelial stem cells in the mouse incisor and elucidated several important regulatory mechanisms that govern the formation and maintenance of these cells. A complex network of signaling pathways like Fibroblast Growth Factor (FGF), vertebrate homologue of the Drosophila Wingless gene (Wnt), (Hedgehog) Hh signaling as well as Notch regulate the formation, maintenance, and differentiation of these stem cells during embryonic development and postnatal stages (Fig. 6)

(Artavanis-Tsakonas et al., 1999; Harada et al., 1999; Harada et al., 2002; Mitsiadis and Graf, 2009; Seidel et al., 2010; Hu et al., 2014).

For instance, FGF10 is required for the development of the cl area and for the formation of the epithelial stem cells niche (Harada et al., 2002). Fgf10-deficient mice die soon after birth, due to an absence of lung development. Since teeth are terminally differentiated during postnatal stages, in order to further study the dental phenotypes of Fgf10-deficient mice, they were transplanted under the kidney capsule of immunocompromised mice. Fgf10 deficient mice, grown under kidney capsules, begin to form roots on the labial side (Yokohama-Tamaki et al., 2006). Additionally, it has been shown that attenuation of FGFR2b signaling leads to impaired growth and failure of the enamel formation (Parsa et al., 2010). Another study has shown that Spry genes, which encode intracellular antagonists of FGF are essential for establishing and sustaining the asymmetry of enamel deposition necessary for normal incisor and that the earliest ameloblasts that form in the incisor are derived from a transient embryonic ameloblast progenitor cell population that does not self-renew (Klein et al., 2008).

Tbx1 expression in developing teeth is under control of FGF signaling and correlates with determination of the ameloblast lineage (Mitsiadis et al., 2008). Deletion of Tbx1 leads to mice with hypoplastic and lacked enamel, while histological results demonstrated the complete absence of ameloblasts. Additionally, the cl area of the mutant incisor was either reduced or completely absent. Therefore, this study concludes that Tbx1 is essential for the maintenance of ameloblast progenitor cells in rodent incisors and that its deletion results in the absence of enamel formation (Catón et al., 2009). Adherens junction molecule E-cadherin has been shown to play an important role in regulating cell proliferation and migration in the continuously growing mouse incisor. Deletion of E-cadherin leads to abnormal morphology of the cl (Li et al., 2012).

Stem cells in the cl area are responsive to Sonic Hedgehog (SHH), which is expressed by their differentiating progeny (Zhao et al., 2014). By using a genetic lineage-tracing assay, it has been shown that Hh-responsive cells are stem cells that can give rise to differentiated progeny (Biehs et al., 2014; Zhoa et al., 2014). Additionally, Hh signaling is required for continued generation of enamel-producing ameloblasts (Seidel et al., 2010).

Epiprofin/Sp6 (Epfn) is essential for the differentiation of dental epithelial progenitor cells into ameloblasts (Jimenez-rojo et al., 2010; Nakamura *et al.,* 2016). Deletion of Epfn results in complete lack of ameloblasts and consequently lack of enamel formation.

Although different molecules have been involved with the generation of the enamel-producing ameloblastic lineage, there is little information about how the other three dental epithelial lineages are established. This may be an important issue to clarify since the lack of some of those accessory epithelial layers may lead to a dysfunctional epithelium and enamel defects (Jimenez-roj*o,* unpublished observations).

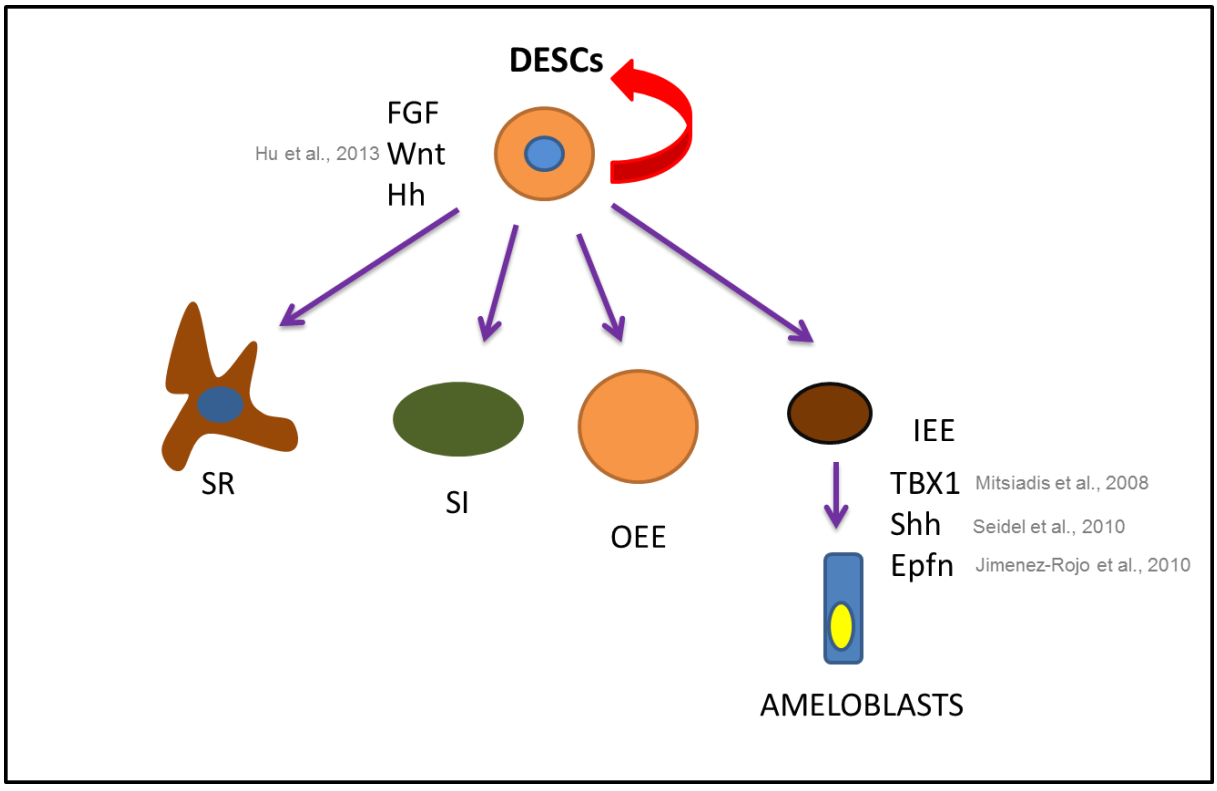


Figure 6: The regulatory network for incisor stem cells. An intricate network of signaling molecules and transcription factors regulates key cellular processes including stem cell maintenance, proliferation and differentiation.

3.5 Notch signaling

In 1913, John Smith Dexter and colleagues observed a notch or indentation at the end of the fly wings. Some years later, in 1917 the alleles of the fly gene were identified and eventually became known as “Notch” (Olsauskas-Kuprys et al., 2013). The group of Artavanis-Tsakonas and Young (Wharton et al., 1985; Kidd et al., 1986) found that Notch signaling pathway is a master regulator of cell fate determination. In invertebrates, Notch activation directs accurate cell fate choices by restricting differentiation towards alternative fates and permits the self-renewal and survival of multipotent cells (Artavanis-Tsakonas et al., 1999).

3.5.1 Notch receptors and ligands

In mammals four Notch receptors have been identified (Notch1-4). Notch receptors are classified as large single-pass type 1 transmembrane proteins composed of the functional Notch extracellular domain (NECD), a transmembrane, and a Notch intracellular domain (NICD). The NECD of each Notch receptor contains 36 Epidermal Growth Factors (EGF)-like domains Notch3 and Notch4 (contain 34 and 26 domains, respectively), a subset of which contains calcium-binding sites and three cysteine-rich Notch/Lin12 domains (LN). Nearby to the NECD is the transmembrane domain with two components: (LN) and heterodimerization domain (HD). The third domain, NICD, which extends from the inner cell membrane into the cytoplasm contains four entities: RAM domain, six Ankyrin repeats, transactivation domain (TAD), a polypeptide proline, glutamate, serine and threonine-rich motifs sequence (PEST). Another difference between the various Notch receptors is that Notch1 contains strong TAD compared to Notch2, which is weaker, but no TAD is found in Notch3 and Notch4. The Notch signaling can be initiated by a receptor-ligand interaction. In mammals five ligands are known: Delta-like (Dll)1, Dll3, Dll4, Jagged-1 and Jagged-2. The ligands contain an amino-terminal structure called DSL (Delta Serrate homologues and LAG-2), followed by 6-16 EGF-like repeats. At the end of the EGF-like repeats a cysteine rich area is located only for the Jagged1 and Jagged2 (Andersson et al., 2011; Olsauskas-Kuprys et al., 2013; Radtke & Raj 2003).

3.5.2 The canonical Notch signaling pathway

The Notch signaling pathway is mediated by regulated proteolysis. Upon translation, the Notch protein is fucosylated on EGF repeats containing the consensus [(C2XXX(A/G/S)(S/T)C3] by the GDP fucose protein O-fucosyltransferase (O-fut1 in Drosophila/Pofut1 in mammals). Additional Notch protein is glycosylated by RUMI, the glycosyltransferase that adds the O-glucose to serine residues in the consensus. These modifications are essential for the production of a functional receptor. The mature receptor is produced after proteolytic cleavage by PC5/furin at Site 1 (S1) and thereafter targeted to the cell surface as a heterodimer held together by non-covalent interactions. Endocytosis and membrane trafficking regulate ligand and receptor availability at the cell surface. Ligand endocytosis is triggered by monoubiquitination mediated by the E3 ubiquitin ligases Neuralized and Mindbomb, which preferentially recognize Delta-type ligands and Serrate/Jagged, respectively. This mechanism pulls Notch to expose it for cleavage at site S2 by ADAM17 or ADAM10 (tumor necrosis factor α-converting enzyme TACE) (Radtke & Raj 2003). The shedding of the Notch ectodomain creates a membrane-tethered intermediate Notch extracellular truncation(NEXT), which becomes a substrate for γ-secretase. This enzyme cleaves NEXT progressively within the TMD (single transmembrane domain) at S3 and S4. This transmembrane aspartyl proteinase, γ-secretase, contains catalytic subunits like presenilin 1 (PS1) or presenilin 2 (PS2), transmemrane proteins nicasterin (NCT) and anterior pharynx defective 1 (APH1). Only NICD allows it to enter the cytoplasm and eventually translocate into the nucleus. The NICD forms a transcriptional complex and binds ubiquitous transcription factor CBF-1, Suppressor of Hairless and Lag-1 (CSL), also known as RBP-jκ in mice. In absence of Notch signaling, CSL associates with ubiquitous co-repressor (CoR) proteins and histone deacetylases (HDACs) and binds to the promoter of its target gene, inhibiting transcription. In the presence of CSL, a large co-repressor complex containing SKI-interaction protein (SKIP), a silencing mediator of retinoid and thyroid hormone receptor (SMRT) and other co-repressors, is displaced. The NIC-CSL complex recruits a co-activator complex containing a Mastermind-like protein (MAML1-3 in mammals), CBP/p300 and other chromatin-modifying enzymes, which leads to transcriptional activation of downstram target genes. Some of the Notch target genes are the helix-loop-helix Hes 1, 5, 6, Hey-L family and c-Myc (Kopan & Ilagan 2009). The

activity of intracellular Notch is terminated by its Ub and subsequent proteasomal degradation (Fig. 7) (Andersson et al., 2011; Olsauskas-Kuprys et al., 2013; Radtke & Raj 2003).

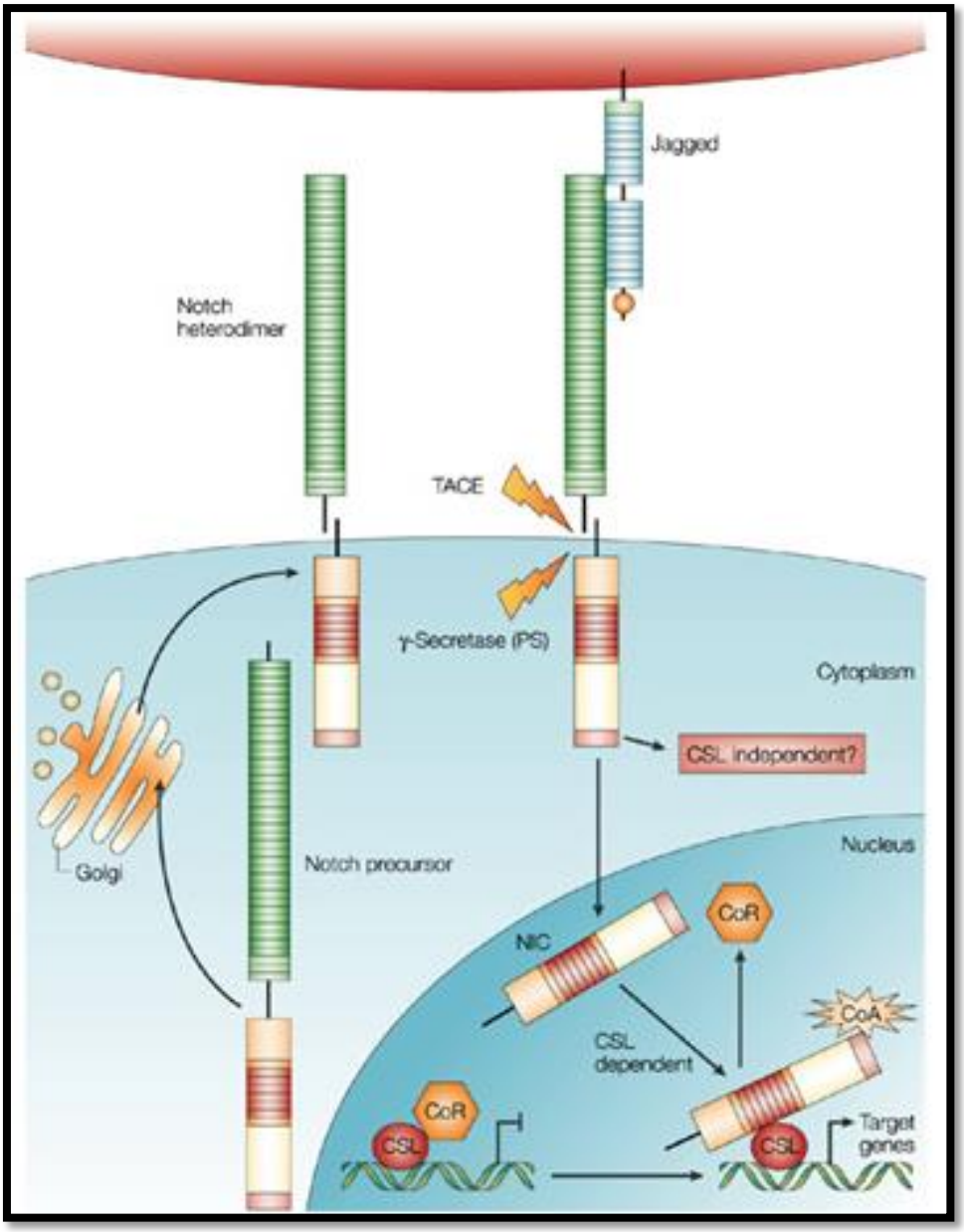


Figure 7. Notch signaling activation: Upon ligand-receptor binding, the extracellular domain of Notch receptors is proteolytically cleaved. Subsequently, the NICD is translocated to the nucleus where it binds to the transcription factor CSL (CBF-1 in humans or RBP-Jk in mice), forming a complex that regulates the transcription of Notch target genes. (Radtke & Raj 2003).

3.5.3 The non-canonical Notch signaling pathway

Notch can also function independent of ligand or transcription, referred to as non-canonical Notch signaling pathway (Andersen et al., 2012, Sanalkumar et al., 2010). Early evidence for CSL-independent non-canonical Notch signaling originated from *in vitro* studies, as it was observed that the inhibition of myoblast differentiation did not require the CLS Notch interacting domain of Notch1 (Nofziger et al., 1999; Bush et al., 2001). Studies in Drosophila *in vivo* revealed that Notch acts independent during muscle precursor development(Rusconi & Corbin 1998). Non-canonical Notch signaling is independent of CSL/RBP-Jκ and, instead, interacts with PI3K, mTORC2, AKT, Wnt, NFκB, YY1, or HIF-1α pathways at either the cytoplasmic and/or nuclear levels (Ayaz & Osborne 2014). Two types of non-canonical Notch signaling pathway have been reported. The non-canonical Notch signaling pathway, Type-I requires ligand mediated cleavage of Notch receptor but change the signals independent of CBF1 interaction. Then, the cleaved NICD interacts either with tissue-specific co-activators (Co-A) and other undefined factors or with components of other signaling pathways in order to activate downstream targets/functions (Fig. 8A). The non-canonical Notch signaling pathway, Type-II Notch target gene ignore either ligand-mediated NICD-release or CBF1-interaction and target genes are activated through other signaling pathways (Fig. 8B) (Sanalkumar et al., 2010).

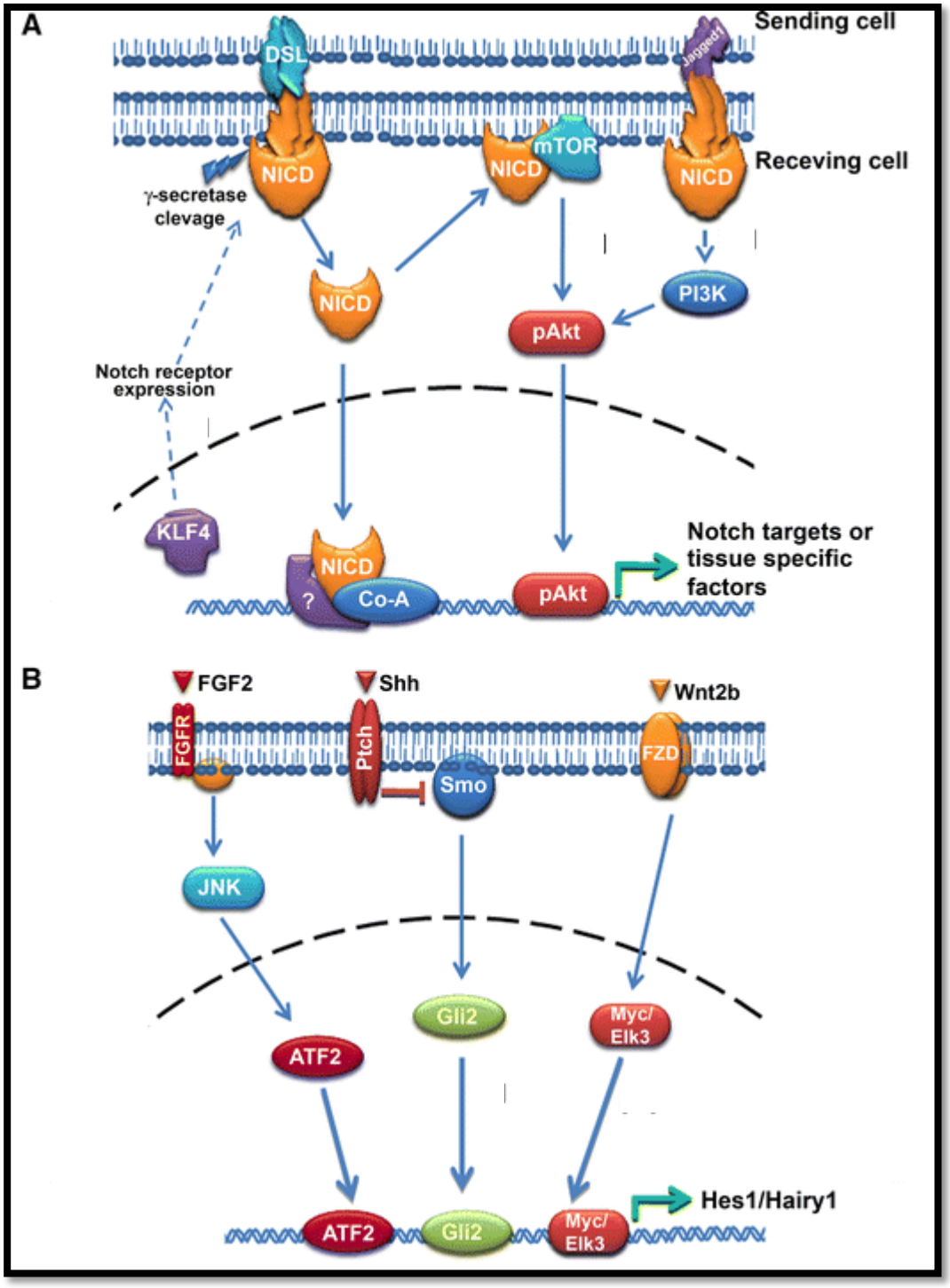


Figure 8. Non- canonical Notch signaling activation: A) Type-I: Ligand mediated cleavage of Notch receptor but change the signals independent of CBF1 interaction. B) Type-II: Notch target gene ignore either ligand-mediated NICD-release or CBF1-interaction and target genes are activated through other signaling pathways. (Sanalkumar et al., 2010)

3.5.4 Notch during development

A major role of Notch is to initiate differential development in adjacent progenitor cells, which ultimately results in complex organs. Many mouse transgenic/mutant lines have been generated for the study of different receptors and ligands of the Notch signaling pathway (Ilagan & Kopan 2014). Mutations in Notch pathway elements affect different organs during the developmental process in mouse. For example, Notch1-/- mice were dying around E10 and defects in somitogenesis as well as vascular remodelling and angiogenesis were observed (Swiatek et al., 1994; Conlon et al., 1995; Krebs et al., 2000; de la Pompa et al., 1997). Notch2-/- mice were dying before E11.5, due to the defects in the kidney, heart and eye development (Hamada et al., 1999; McCright et al., 2001). Notch3 deficient mice were developed normally although the maturation of arterial smooth muscle cells was affected (Domenga et al. 2004; Krebs et al. 2003). Jagged1 deficient mice died around E10 and vascular and ear defects were observed (Xue et al., 1999; Kiernan et al., 2001). Jagged2-/- mice perinatal lethality and defects in the limb and thymic development were observed (Jiang et al., 1998; Sidow et al., 1997). Delta like 1 (Dll1) deficient mice were dying around E12 and brain defects as well as reduce neurogenesis and gliogenesis (Hrabě de Angelis et al., 1997; De Bellard et al., 2002). Dll3 deficient mice died postnatally between P0 and P10 and Dll4 -/- mice were dying because of defects in vascular remodelling. (Kusumi et al., 1998; Duarte et al., 2004; Krebs et al., 2004). Disruption of RBP-Jk kappa gene results in early embryonic death like E8.5 as well as postimplantation, somatic and vascular development defects were observed (Oka et al., 1995; Krebs et al., 2004). Mice deficient for Hes1, Hes5, Hes7, Hey 2 die between E10.5 and E15.5 due to defects in the different organs during development (Zine et al., 2001; Hirata et al., 2001; Kokubo et al., 2005; Kokubo et al., 2004; Zhong et al., 2000). Lethality were observed also with mice deficient with Presenilin 1, Presenilin 2 and furin (Wong et al., 1997; Koizumi et al., 2001; Wines-Samuelson et al., 2005; Donoviel et al., 1999; Roebroek et al., 1998). Since most of the mutant mice carrying complete deletion of Notch component are lethal during embryogenesis or soon after birth, conditional knockout mouse models are usually necessary.

3.5.5 Role of Notch signaling in cell fate determination

Notch pathway is a master regulator of cell fate determination and permits the self-renewal and survival of multipotent stem cells in ectoderm-derived organs (Artavanis-Tsakonas et al., 1999). For example, by studying Msx2N1N2cKO mice that are invalidated for both *Notch1* and *Notch2* in Msx2-expressing cells, it has been shown that Notch controls differentiation and homeostasis in hair follicles (Pan et al., 2004). Specifically, for the Notch1 receptor it has been demonstrated that it is essential for late stages of hair follicle development during embryogenesis as well as for postnatal hair follicle development and hair homeostasis (Vauclair et al., 2005; Vauclair et al., 2007).

In the mammary gland, by using the report mice Notch1CreERTmT/mG it has been shown that Notch1 positive cells are multipotent stem cells during embryonic stages, which then switch to unipotent stem cells postnatally (Rodilla et al., 2015). These cells have extensive self-renewal capacity; they preserve their identity throughout adult life and are highly responsive to hormones. Importantly, they can repopulate the entire mammary gland in transplantation assays, especially when pregnancy-induced hormones stimulate them (Rodilla et al., 2015).

There is evidence that has shown that the Notch signaling is not only key regulator of cell fate determination during development and tissue homeostasis but also during tissue repair processes of different organs. For example, it has been observed that Notch1 signaling is essential for tissue repair of the corneal epithelium after injury (Vauclair et al., 2007). Notch signaling appears to be important for hematopoietic stem cell regeneration, after myeloablation from lethal irradiation. Additionally, conditional deletion of RBP-J, which is essential for mediating canonical Notch signaling, lead to reduction of the muscle stem cells and failure of tissue regeneration after muscle injury (Philippos et al., 2012). The mammalian epidermis consists of regions that contain hair follicles interspersed with interfollicular epidermis. Hair follicle morphogenesis relies on both quiescent and active stem cells. Interestingly, it has been shown that adult epidermal homeostasis regulated by active stem cells and the quiescent stem cells seem to rely solely after injury-healing process (Clayton et al., 2007; Rompolas et al., 2012). Finally, yet importantly, the intestinal epithelium has the ability to be renewed every 5 days. It has specific progenitor cells that display plasticity and can regain stemness upon tissue damage, by using *Dll1*$^{GFP\text{-}ires\text{-}CreERT2}$ knock in mice (van Es et al.,

2012). Recently, it has been shown that Notch1 is the primary receptor regulating intestinal stem cell function and that Notch1 and Notch2 together regulates epithelial cell proliferation, cell fate determination, and post-injury regeneration (Carulli et al., 2016).

In the developing tooth, Notch signaling has been proposed as an important regulator of dental cell type specification in the enamel organ (Mitsiadis & Graf 2009). However, the role of Notch signaling pathway in the regulation of dental epithelial stem cell (DESCs) fate still remains unclear.

3.5.6 Notch in tooth

It is well established that Notch signaling plays an important role during odontogenesis. Previous studies have shown that different Notch receptors and ligands are expressed in the different layers of the epithelium. Between E11 and E12 stages, Notch transcripts are expressed in the dental epithelium but are absent from cells of the basal layer. At E13 all the three genes are expressed in the epithelium and at E14.-E15 Notch1 and Notch2 are expressed in the cells of the enamel organ while the Notch3 are expressed in vascular structures. At E15-E16, all three Notch genes are expressed in the enamel organ. Specifically, Notch1 are expressed in the inner mass, Notch2 in the sr layer, Jag1 in the enamel organ and Jag2 in the intermediate layer. In the bell stage E16-E18, Notch1 and 3 are found in si, whereas Notch 2 transcripts are detected in si, sr and oee (Fig. 9) (Mitsiadis et al., 1995; Mitsiadis et al., 1997; Mitsiadis et al., 1998; Mitsiadis & Graf 2009). The expression pattern of the Notch genes is the same in the mouse incisor at E18.5, where the Notch1 and Notch3 are also expressed in the cl area (Fig. 10) (Mitsiadis et al., 1998). Postnatally (P1) the Notch1 is expressed in the cl area and becomes restricted to the si and sr in the other areas that follow cl (preameloblasts, secretory ameloblasts, mature ameloblasts). Notch1 was not observed to be express in the oee and ameloblasts (Fig. 10) (Natsiou, unpublished data). Another study showed that recombinant Jag1 protein added to HAT-7 dental epithelial-like cells in culture enhanced the appearance of cells resembling the si layer (Yokohama-Tamaki et al., 2006).

Additionally, by inhibition of *Jagged1*, *Jagged2*, *Notch1*, and *Notch2* alone and in combination led to defects in the ameloblast-si interface and, ultimately, enamel formation (Jheon et al., 2015). As previously mentioned, incisor allows us to visualize all the cell lineages in one

individual. Thus, mouse incisor is a great model to trace Notch1 positive epithelial cells and their progeny during homeostasis and regeneration.

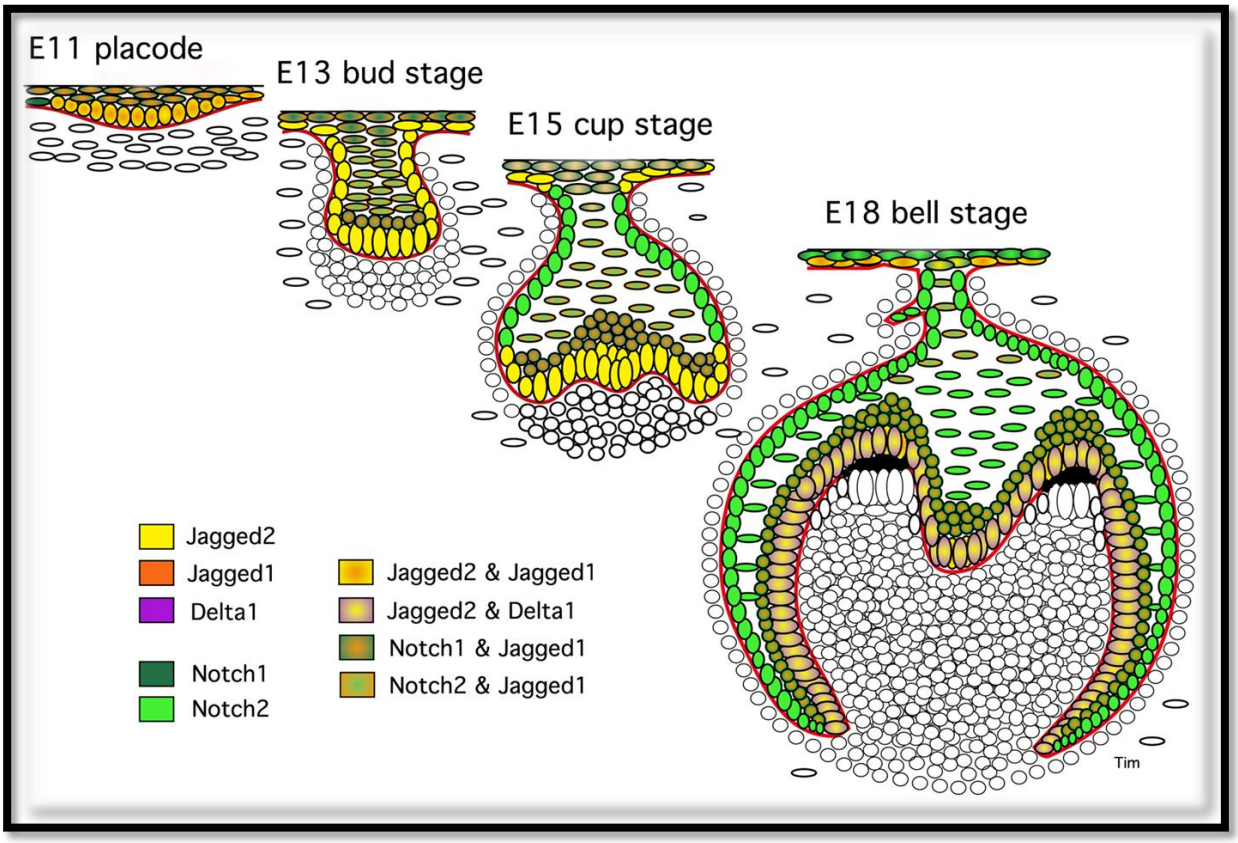


Figure 9.Expression of different receptors of Notch signaling in the developing teeth. Different Notch receptors (in the boxes) and combination of receptors and ligands (combination colours) show expression in each cell layer during organogenesis. Mitsiadis and Graf., 2009

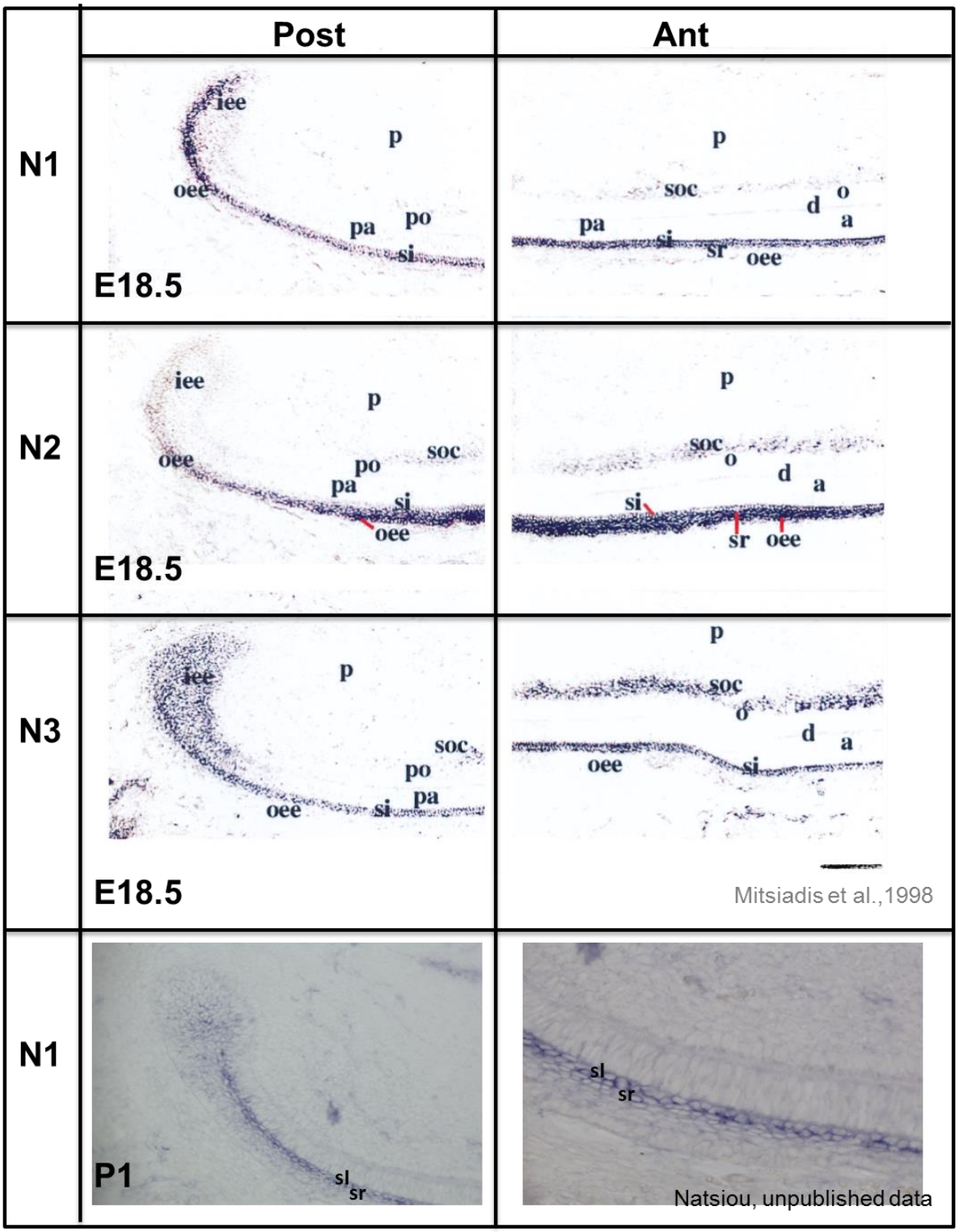


Figure 10. Expression of different receptors of Notch signaling by *in situ* hybridization at E18.5 and postnatally. Different Notch receptors show expression in each cell layer at E18.5 incisor., 2009. Notch1 is expressed in the cl and si/sr in the differentiation areas of P1 incisor. Mitsiadis and Graf., 2009; Natsiou, unpublished data.

4. Aims of the project

The aim of this study is to examine the role of Notch signaling in fate determination of dental epithelial stem/progenitor cells. This has been accomplished by combining both *in vitro* and *in vivo* methods in order to assess the specific role of Notch signaling, and more specifically Notch1, during the establishment of dental epithelial cell lineages.

Therefore, the project follows three principal aims:

4. 1. To elucidate the role of Notch in the regulation of dental epithelial stem cells *in vitro*.

First of all, the optimal conditions for the three-dimensional (3D) *in vitro* culture of dental epithelial stem/progenitor cells will be stablished. To achieve that, we aimed to develop a validated protocol by using different growth factors in order to obtain epithelial dentospheres. Then, by using this model, the effect of the inhibition of Notch signalling in the formation of the dentospheres will be assessed by using the γ-secretase inhibitor DAPT.

4. 2 To perform lineage tracing of Notch1-expressing cells in dental epithelium.

By using the reporter mice N1CreERTmT/mG, we will follow the progeny of Notch1-expressing cells from dental epithelium during:

4.2. a Embryonic development. We will use organotypic culture assays *in vitro* in order to trace the fate of Notch1 positive cells during tooth development. Labelling of Notch1-expressing cells will be performed in E14.5 incisors and they will be cultured in semi solid plates and analysed at different time points.

4.2. b Postnatal stages. We will trace the fate of Notch1 positive cells in postnatal incisors.

4.2. c Regeneration. We will investigate the role of Notch1 positive cells during regeneration following injury in the postnatal incisor.

4. 3 To elucidate the role of Notch1 in cell fate determination of dental epithelial stem cells *in vivo*.

The effects of Notch1 deletion in dental epithelium will be studied by using conditional knock out K14CreN1$^{flox/flox}$ mice (K14N1cKO).

5. Materials and methods

5. 1 Mice

All mice were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary office, Zurich.

Postnatal K14CreNotch1fl/fl (Li et al., 2001;Radtke et al., 1999) in a 129S2/SvPasCrl background were analysed. Briefly, Tg(KRT14-cre)1Ipc referred as K14Cre mice (Li et al., 2001) were crossed with Notch1tm1Agt referred as Notch1$^{flox/flox}$ mice (Radtke et al., 1999) in order to specifically delete Nocth1 expression in dental epithelium. Additionally, in order to check the efficiency of the Notch1 deletion, *K14CreNotch1$^{fl/fl}$*, backcrossing with the Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J reporter mice that was obtained by the Jackson Laboratories. Plug days were taken as E0 and embryos were staged according to morphological criteria. The day of birth was considered as postnatal 0 (P0).

The generation of Notch1CreERT mice has been previous described (Fre et al., 2011). These mice were crossed with the Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J reporter mice. In order to trace the lineage in postnatal stages, postnatal day 3 (P3) Notch1CreERTmT/mG mice were injected with 0.1mg/g of Hydro-tamoxifen (4-OHT) either one time or 2 consecutive times and the mice were analysed after one day, one week, and one month. In order to trace the lineage during regeneration *in vivo*, Notch1CreERTmT/mG mice, including control mice, were intra-peritoneally injected with 50 mg/kg 4-OHT 3 consecutive times followed by trimming of the incisor. Complete regrowth of the incisor was observed after 1 week.

Immunocompromised mice RAG1$^{-/-}$ were used as recipients for the kidney capsule transplantation.

Genomic DNA was extracted from either ear tip or tail tip and was analysed by Polymerase Chain Reaction (PCR) with the following primers:

Notch1: CTGAGGCCTAGAGCCTTGAA, TGTGGGACCCAGAAGTTAGG

Floxed band 500bp, wt band 450bp.

Cre:GCGGTCTGGCAGTAAAAACTATC,GTGAAACAGCATTGCTGTCACTT,
CTAGGCCACAGAATTGAAAGATCT, GTAGGTGGAAATTCTAGGCATCATCC

Transgene 100bp, internal positive 324bp

5.2 Isolation of Dental epithelial stem cells

The incisors were dissected from P6 mice by breaking the alveolar bone and then they were incubated 20 minutes with digestion buffer for 20 min at RT in Dispase (2 mg/ml) and DNAse (20 U/ml) solution in Hanks' Balanced Salt Solution (HBSS) (Fig. 11A). The cl was enzymatically and mechanically separated from mesenchyme (Fig. 11B). Harvested cls were placed in Phosphate buffer saline (PBS) /10% Calf serum (CS) in order to stop the enzyme activity and they were centrifuged at 300G for 5min and supernatant was discarded. Cells were dissociated with 0.25% Trypsin in PBS for 30min at 37 ^{o}C by mixing them gently and further incubating them for 5min with DNase I (400U/ml). Then cell suspension was sieved through a 40um cell strainer (Falcon). Single dental epithelial cells were cultured in a CO_2 incubator at 37^{o}C with fresh culture media containing different combinations of growth factors. The culture medium was either the Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco) containing 2% B27 (Gibco), 100units/ml Penicillin/Streptomycin (P/S) (Gibco), 10µg/ml Heparin (Stem cells Technologies), 10µg/ml Insulin (Sigma), and the growth factors was 20ng/ml EGF (Invitrogen), 20ng/ml FGF2 (Gibco) and 20ng/ml FGF10 (Pepro tech) or the Keratinocyte serum-free medium (KSFM) with Bovine Pituitary Extract (BPE) and EGF (Gibco). Cells were plated in ultralow attachment plates at a density of 20.000 viable cells/ml either alone or in combination with Matrigel. The medium was replenished every two days.

When Matrigel was used, it was digested with dispase 1mg/ml for 30min at 37 ^{o}C prior to harvesting the formed spheres.

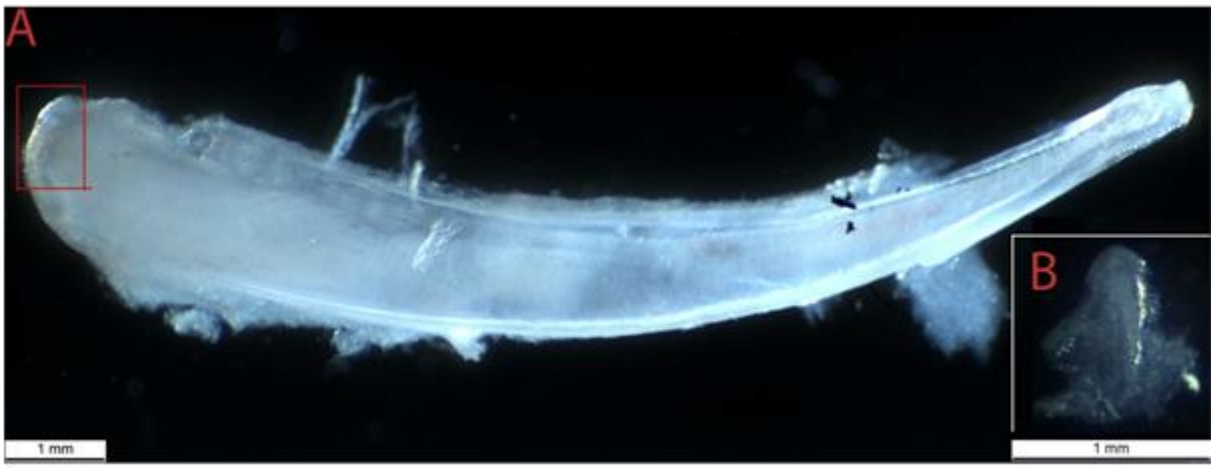


Figure 11: Light microscopy picture of dissected incisor. A) Incisors from P6 mice were dissected B) and the cervical loop epithelium was isolated. Scale bar A-B: 1mm.

5.3 Tooth organ cultures

Teeth were carefully dissected from the lower jaws of N1CreERT2-R26mT/mG mice. Teeth were then placed on top of a 0.1-0.8 µm Whitman filter on a stainless-steel wire mesh (0.25 mm diameter wire) in an organ culture dish containing medium composed of DMEM (high glucose 4.5mg/ml) (GE Healthcare, UK), 20% Fetal Bovine Serum (Pansera, Germany), L-Glutamine, 20 U/ml penicillin/streptomycin and 0.9mM ascorbic acid and were cultured in a humidified atmosphere of 5% CO_2. In order to activate the Cre recombinase the samples were incubated either for 30 mins or overnight with 5µM of 4-OHT. At the desired time poits of analysis, samples were fixed in 1% paraformaldehyde (PFA) at 4^{o}C for 1hour then processed for confocal analysis.

5.4 Tooth injury

Tooth injury was performed using three different procedures in order to assess the potential of Notch1-expressing cells during regeneration:

1. The first procedure was to perform an injury in the cl area were the stem/progenitors cells reside. After dissection of P3 incisors, Cre was activated by exposing the incisors to 4-OHT overnight, then injury was performed in the edge of the cl area with a syringe needle. The intact incisor was used as a control (Fig. 12A).

2. For the second approach, incisors of adult mice were trimmed. After three consecutive 4-OHT injections in postnatal mice, injury was performed by trimming the erupted part of the incisor (Fig. 12B). Complete regrowth of the incisor was observed after seven days. As a control, uninjured incisor was used.

3. The third procedure consisted on cutting the posterior part of postnatal incisor followed by transplantation of the tissue under the kidney capsule of immunocompromised mice. Intact incisors were used as control when mice up to 2 weeks-old were used. Ten days after transplantation under the kidney capsule, the samples were harvested and processed for confocal analysis (Fig. 12C).

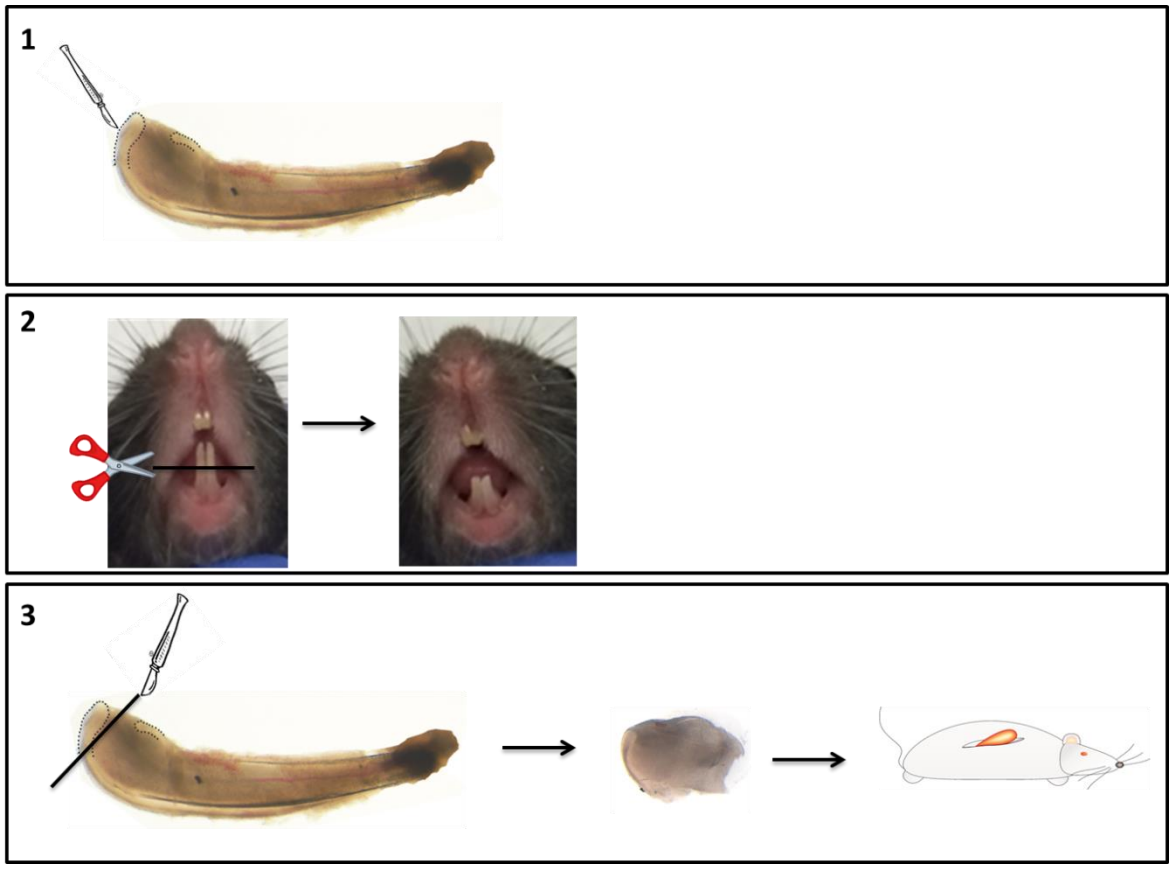


Figure 12: Schematic representation of the different procedures to perform tooth injury. 1) Making an injury in the cl 2) trimming the incisors of adult mice 3) cutting off the posterior part and transplanting it under the kidney capsule of immunocompromised mice.

5. 5 Tissue collection

Embryonic and postnatal tissues for histological and confocal analyses were dissected in cold PBS and fixed overnight in 4% paraformaldehyde (PFA) and 1% PFA for 1 hour respectively. Adult mice were perfused intracardially with 4% PFA. For histology, tissues were decalcified for 1-8 weeks in 10% ethylenediaminetetraacetic acid (EDTA), dehydrated, embedded in paraffin and sectioned at 5 μm for immunochistochemistry, immunonofluoresence and hematoxylin/eosin staining. For *in situ* hybridization, the tissue was embedded in OCT and sectioned at 12μm. Decalcification was not performed on embryonic tissues.

5.4 Hematoxylin and Eosin staining

Hematoxylin/Eosin (H&E) stanings were performed on paraffin and cryostat sections. Rehydrated sections were incubated for 30 seconds in filtered hematoxylin, washed with tap H_2O, incubated for 1' in eosin, dehydrated and mounted with Eukitt medium.

5.5 Prussian blue staining

Prussian blue staining was performed on paraffin sections. Rehydrated section were incubated for 20min with equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide, washed with tap water, incubated with nuclear fast red for 5min and after dehydration the sections were mounted with Eukitt.

5.6 In situ hybridization

In situ hybridization was performed on cryostat sections as previously described (Mitsiadis et al., 1995b). 400 bp fragment of mouse cDNA were subcloned into pBlueScriptII SK plasmid. Digoxigenin-UTP-labeled single-stranded antisense RNA probes were prepared by standard procedures. The plasmid vectors were linearized with, BamHI for ameloblastin (AMB) and EcorI for amelogenin (AMG) (Promega). *In situ* hybridization was performed via incubation with the probe at 63°C overnight. After post-hybridization washes, the slides were incubated in blocking solution (MABT + 20% Normal Goat Serum) and incubated with Anti-digoxigenin

AP- alkaline phosphatase conjugate Fab fragment (Boehringer Mannheim, 1093 274) diluted 1:1000 in blocking solution. The colour reaction was developed using Nitro Blue Tetrezolium (Sigma N-6876) and 5-Bromo-4-Chloro-3-Indolyl Phosphate, (Sigma B-8503) in staining solution 100Mm NaCl, 50mM MgCl2, 100mM Tris-HCl (pH 9.5), ans 0.1% Tween-20.

5.7 Immunofluorescence

Sections w de-waxed and rehydrated through xylene and gradient of ethanols and subjected to antigen retrieval by microwave heating in 10mM Trisodium Citrate buffer (pH 6.0). The tissue was then permeabilized with 0.5% Tween-20; non-specific antibody binding was blocked with 1% BSA for 1 hour and then sequentially incubated with primary antibody overnight at 4^{o} C and then followed by incubation in the secondary antibodies goat anti-rabbit (Life Technologies Europe, Switzerland) for 1 hour at Room temperature. The following primary antibodies were used: K10 (1:100, BioLegend*), Amg* (1:100, Abcam), Sox2 (1:50 Abcam) and GFP (1:100, Invitrogen, Thermo Scientific). With DAPI staining was visualized the nuclei. Pictures were taken using the Leica DM6000 FS microscope, the Leica DFC350FX camera and the Leica Application Suite (LAS) software (Leica Microsystems AG, Heerbrugg SG, Switzerland).

5.8 Backscattered Scanning Electron Microscopy (SEM) analysis

Fully mineralized lower hemi-jaws were dissected from perfused 4 and 8 week-old adult K14N1cKOand wild-type (WT) mice. After incubation of t the samples with 3% hydrogen peroxide (H_2O_2) overnight the soft tissues were removed the following day. The lower jaws were dehydrated and embedded in Technovit 7200 VLC (Heraeus Kulzer, Wehrheim, Germany). Light-polymerized blocks were mounted on aluminium stubs, polished and coated with a 10-15 nm thick layer of carbon. By using a Tescan EGATS5316 XMSEM (Tescan, Brno, Czech Republic) operated in BSE mode were examined. Elemental composition of enamel was analysed with the aid of energy-dispersive X-ray spectroscopy. A Si(Li) detector (Oxford Instruments, Wiesbaden, Germany) served for recording EDS spectra using an accelerating voltage of 7 kV, a working distance of 23 mm, and a counting time of 100 s.

5.9 Transplantation assay

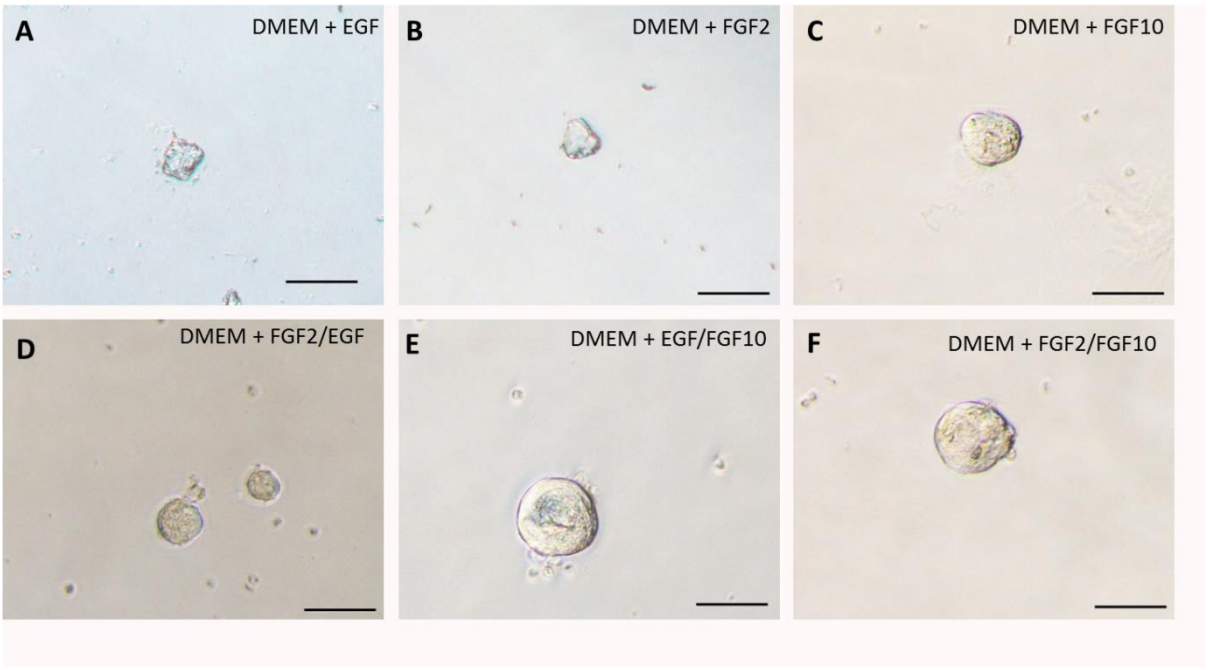
Eight to ten week-old immunocompromised mice (RAG1-/-) were used as recipients for the transplantation assay. Buprenorphine (0.1 mg/kg bodyweight) was subcutaneously injected before the surgery. Mice were anaesthetised by isoflurane inhalation. The kidney was exposed by cutting the back skin and perinoneum. The membrane (capsule) that was covering the kidney was gently opened in order to make an access point to introduce the donor tissues. After placing the transplanted tissues under the kidney capsule, the kidney was placed back to the normal position and the peritoneum sutured. Mice were put onto a warming pad and observed until they reached consciousness. For pain management, overnight, we orally administrated Buprenorphine via the drinking water (buprenorphine 0.3 mg/ml dissolved in 160 ml of water). Ten days after the transplantation, the mice were euthanized and the kidney was carefully dissected and processed for analysis.

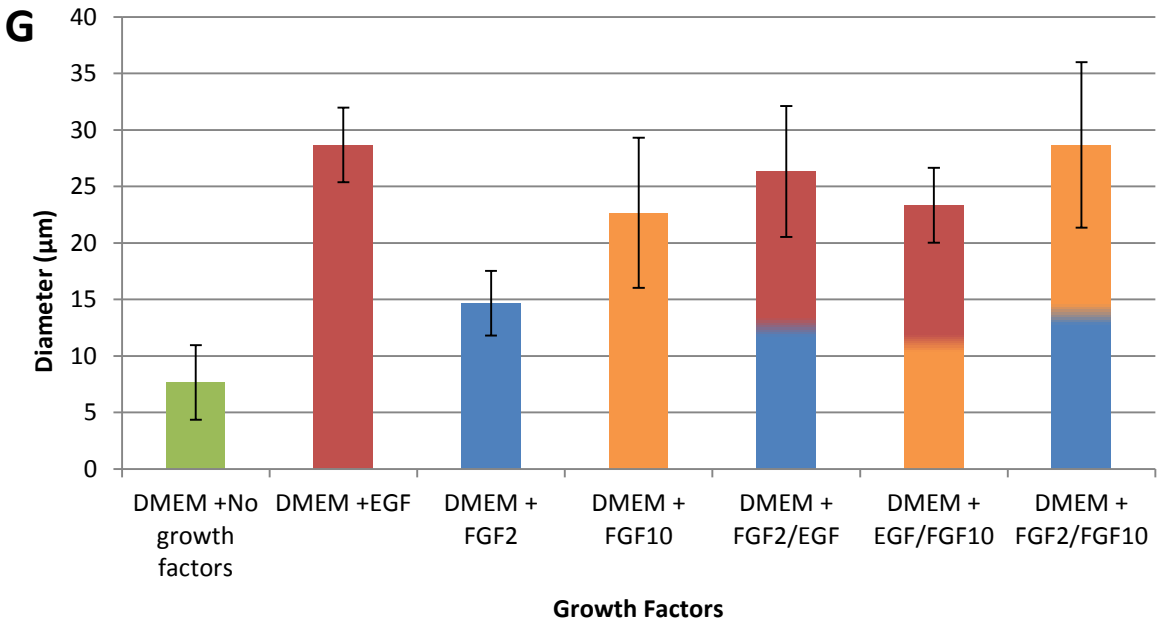
6. Results

6.1 Role of Notch in the regulation of dental epithelial stem cells in vitro

6.1.1 Optimization of 3Dimensional (3D) culture *in vitro* of Dental Epithelial Stem Cells (DESCs).

In order to optimize the sphere-assay, isolated dental epithelial cells from the labial cl area from P6 mouse incisor were cultured *in vitro* under ultra-low attachment conditions. In ultra-low attachment plates isolated single cells were cultures with medium and combination of different growth factors like Epidermal Growth Factor (EGF), Fibroblasts Growth factor (FGF2), FGF10, FGF2/EGF, EGF/FGF10 and FGF2/FGF10 (Fig. 13). Results showed that when the sphere medium contained growth factors EGF and FGF2 alone (Fig. 13A-B), there was not dome-shaped sphere formation. On the other hand, spheres were generated by incubating the DESCs with the FGF10 as well as the combination of the growth factors. Additionally, in order to further investigate the optimal conditions for dentosphere formation; we measured the sphere diameter (μm) by using Fiji software (Fig. 13G). Results indicate that the combination of FGF2/EGF and FGF2/FGF10 can form dentospheres with larger diameters, compared to the other conditions with the different growth factor combinations. Furthermore, the number of dentospheres formed for each condition was counted by using Fiji software. Results showed very small number of dentospheres when the cells where cultured without any factor and very large number of dentospheres when the cells were cultured with EGF, EGF/FGF2 and FGF2/FGF10 (Fig. 13H).


A
DMEM + EGF
B
DMEM + FGF2
C
DMEM + FGF10
D
DMEM + FGF2/EGF
E
DMEM + EGF/FGF10
F
DMEM + FGF2/FGF10


G
40
35
30
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10
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0
Diameter (µm)
DMEM +No growth factors
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DMEM + FGF2
DMEM + FGF10
DMEM + FGF2/EGF
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DMEM + FGF2/FGF10
Growth Factors

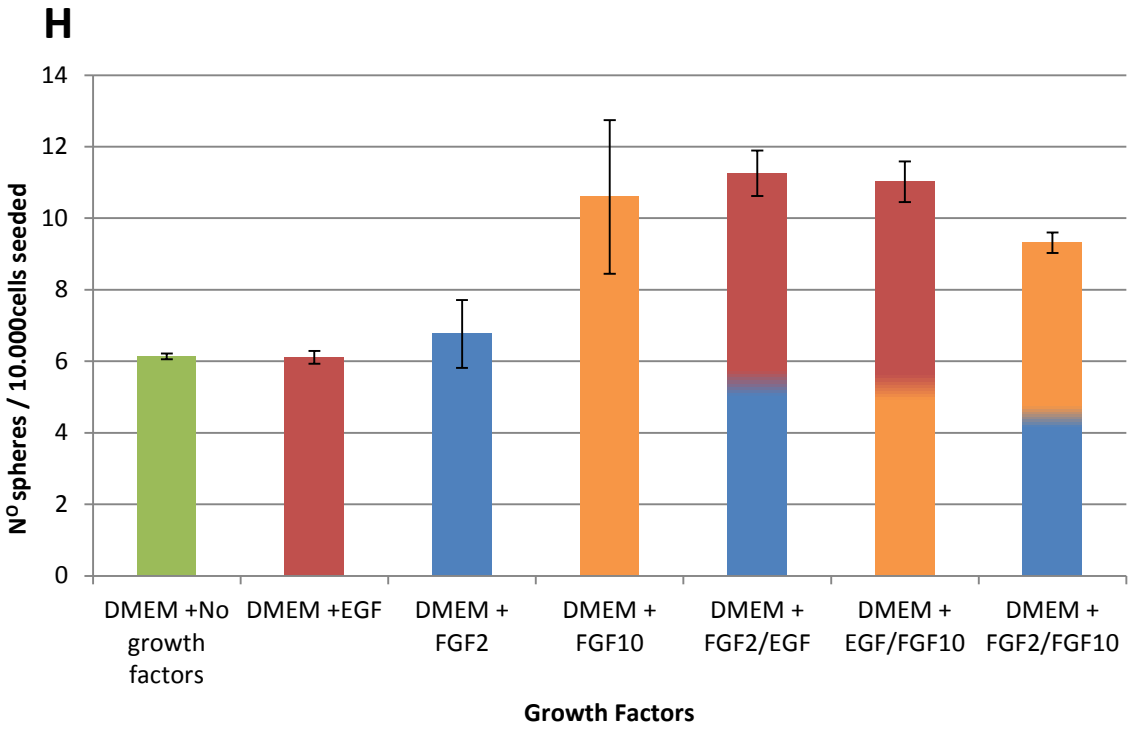
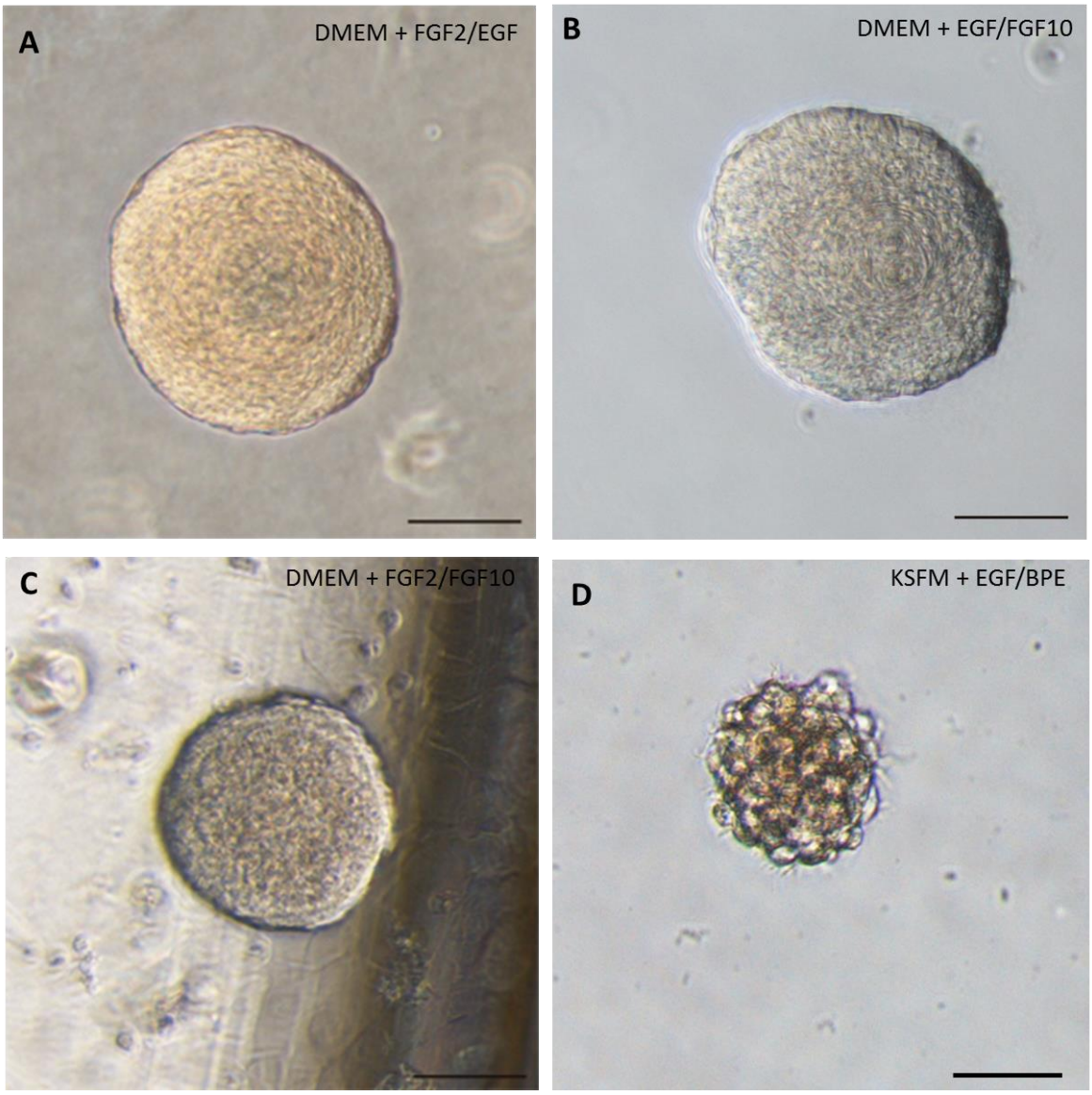


Figure 13. Dentospheres formation. No dome-sphere formation was observed when the medium contains A) EGF B) FGF2 growth factor. Dentospheres formation was observed when the sphere medium contains C) FGF10 D) FGF2/EGF E) EGF/FGF10 and F) FGF2/FGF10. Growth factor combination determines the G) diameter and the H) number of formed dentospheres. Abbreviation: DMEM: Dulbecco's Modified Eagle Medium, EGF: Epidermal growth factors, FGF: Fibroblast Growth factor, Error bars: Mean±SD, Scale bar: 20µm

Then, we cultured the cells with Matrigel at 1:1 ratio and either Dulbecco's Modified Eagle Medium (DMEM) or Keratinocyte serum-free medium (KSFM) medium with the combination of different growth factors. Results showed the formation of epithelial dentoshperes with both mediums and combination of growth factors (Fig. 14A-D). Quantitative analyses showed that DMEM and FGF2/EGF growth factors can form spheres with larger diameters when compared to the other conditions (Fig. 14E). Additionally, the average of the sphere formation per 10.000 seeded cells was around 18 with DMEM and FGF2/EGF, 9 with DMEM and EGF/FGF10, 7 with DMEM and FGF2/FGF10 and 19 with KSFM and EGF/ (Bovine Pituitary Extract) BPE growth factors (Fig. 14F).


A
DMEM + FGF2/EGF
B
DMEM + EGF/FGF10
C
DMEM + FGF2/FGF10
D
KSFM + EGF/BPE

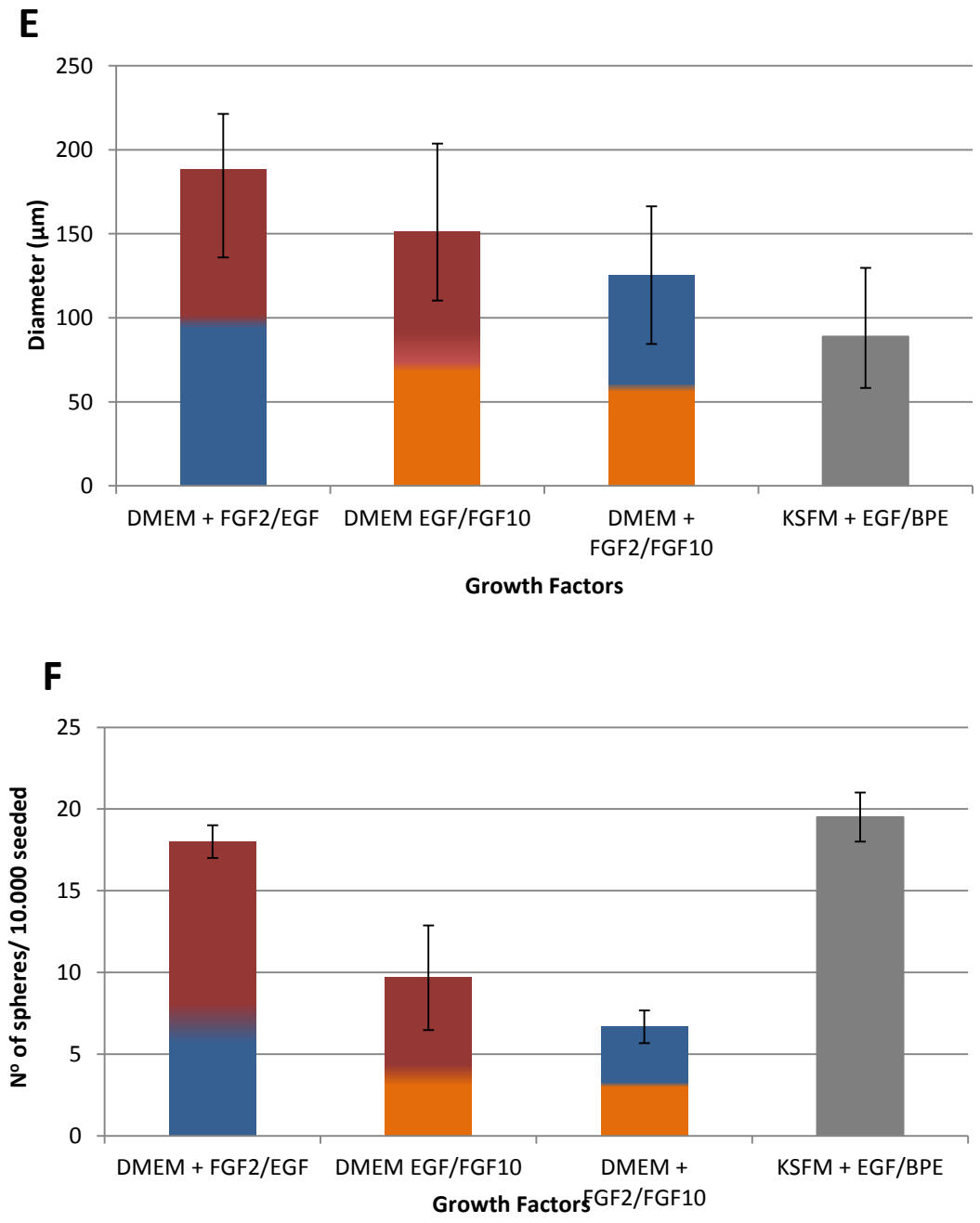


Figure 14. Dentospheres formation with Matrigel. Dentospheres formation was observed when cells were treated with A) DMEM + FGF2/EGF B) DMEM + EGF/FGF10 C) DMEM + FGF2/FGF10 D) KSFME + EGF/BPE. Medium and growth factor combination determines the E) diameter and the F) number of formed dentospheres. Abbreviations: BPE: Bovine Pituitary Extract; DMEM: Dulbecco's Modified Eagle Medium, EGF: Epidermal growth factor, FGF: Fibroblast Growth factor; KSFM: Keratinocyte serum-free medium. Error bars: Mean±SD, Scale bar: 50µm.

6.1.2 Characterization of the dentospheres

In order to characterize dentospheres, we performed H&E staining on paraffin sections. The dentospheres were observed to have a round shape in all the conditions (Fig. 15A-C). However, the forming spheres that were treated with DMEM medium and different growth factors presented a multilayered structure with an acellular center. Interestingly, the center of the spheres was positive against the epidermal differentiation marker Keratin 10 (K10) (Fig. 15E-G).

By contrast, spheres formed in presence of KSFM and EGF/BPE growth factors presented a mass-like structure with cells homogeneously organized (with no clear layers) (Fig. 15D) and they were K10 negative (Fig. 15H). Additionally, the dentosphere were Sox2 positive (Fig. 16).

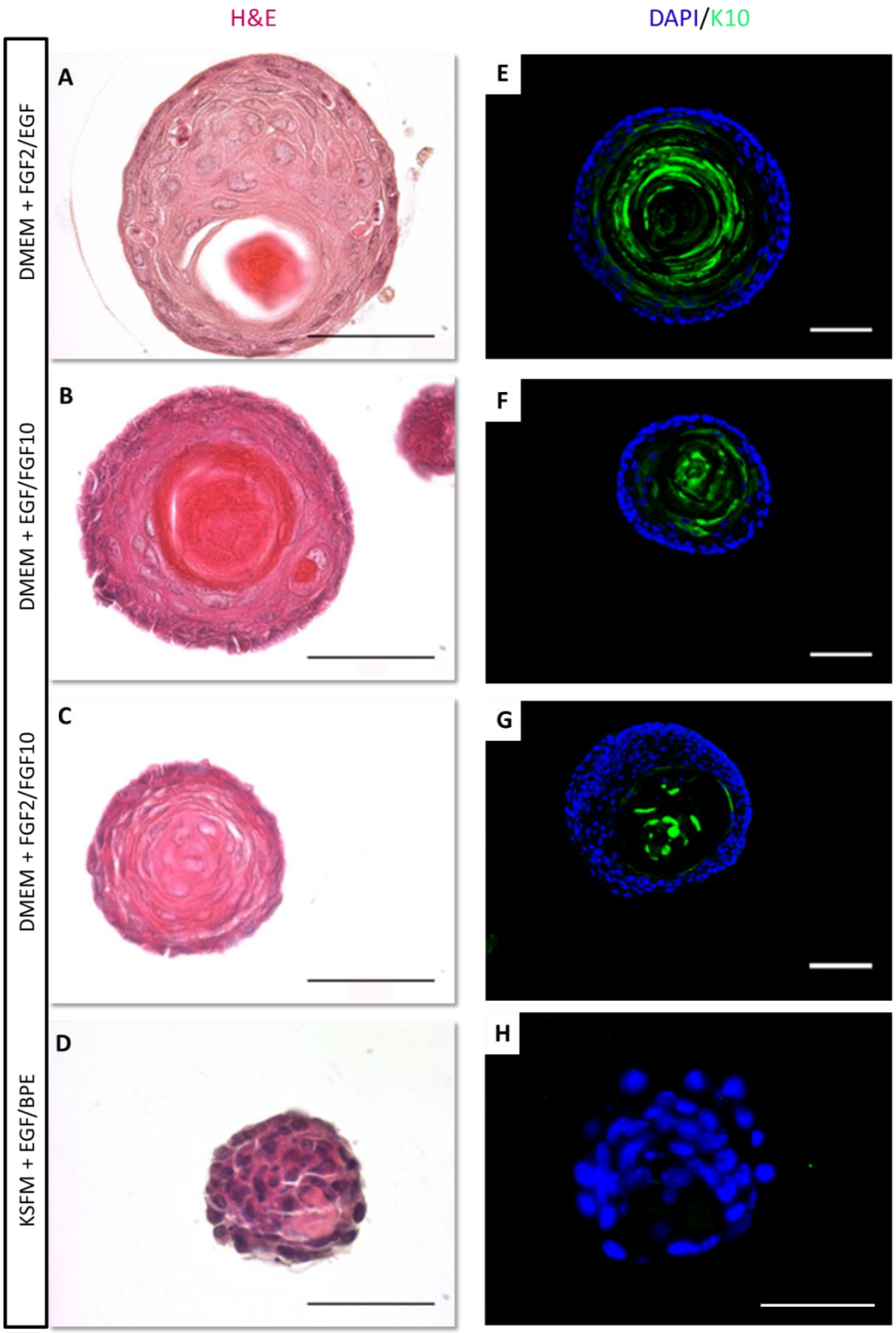


Figure 15.Characterization of the dentospheres. A-D) H&E staining of the forming dentospheres that were treated with different growth factors. E-H) Immunofluorescence against K10. Abbreviations: BPE: Bovine Pituitary Extract; DMEM: Dulbecco's Modified Eagle Medium; EGF: Epidermal growth factors; FGF: Fibroblast Growth factor; KSFM: Keratinocyte serum-free medium. Scale bars:50µm.

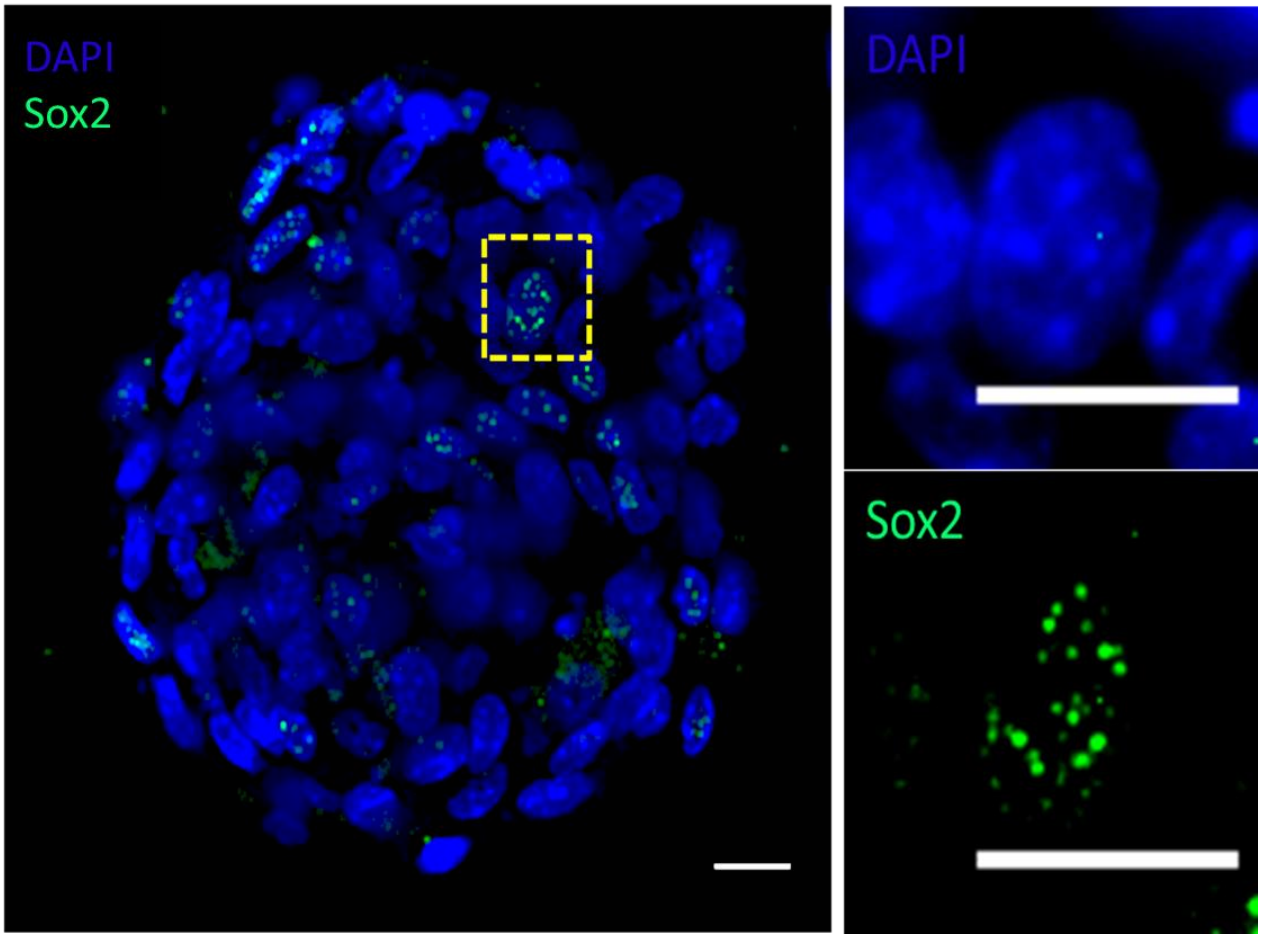


Figure 16. Dentosphere express Sox2. Immunofluorescence against Sox2 of the forming dentosphere treated with KSFM plus EGF/BPE. Scale bars: 10µm.

6.1.3 Effect of Notch signaling inhibition in the dentospheres

As we optimized a validate protocol, we used the KSFM with EGF/BPE medium, in order to check the effect of Notch signaling inhibition in the dentosphere formation. Cells were treated with γ-secretase inhibitor, the N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl ester (DAPT) (5µM) and as a control we used cells treated with the vehicle solution DMSO. After 10days, results showed formation of dentospheres in cells treated with DAPT as in the ones treated with vehicle solution (Fig. 17A-B). Quantitative analysis revealed lower number of dentosphere formation treated with DAPT (Fig. 17C). Spheres treated with DAPT showed an acellular area that was not expressing (Keratin 10) K10 and Amelogenin (*Amg)* (Fig. 18).

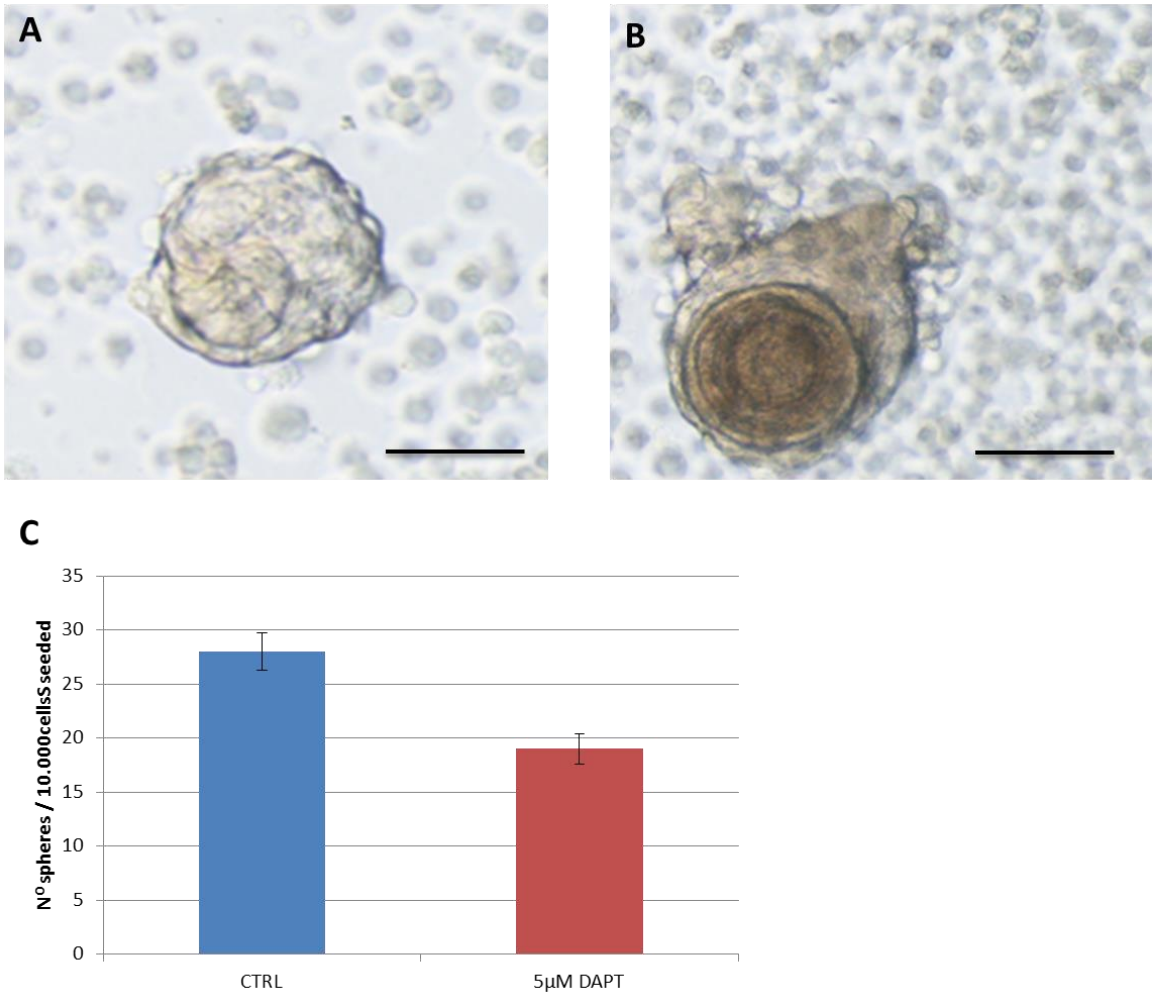


Figure 17.Effect of Notch signaling inhibition in the formation of epithelial dentospheres. Control cells (A) and cells treated with DAPT formed spheres. C) Cells treated with DAPT resulted in less spheres formation. Abbreviation: DAPT: (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl ester), CTRL: Control. Scale bar: 50µm.

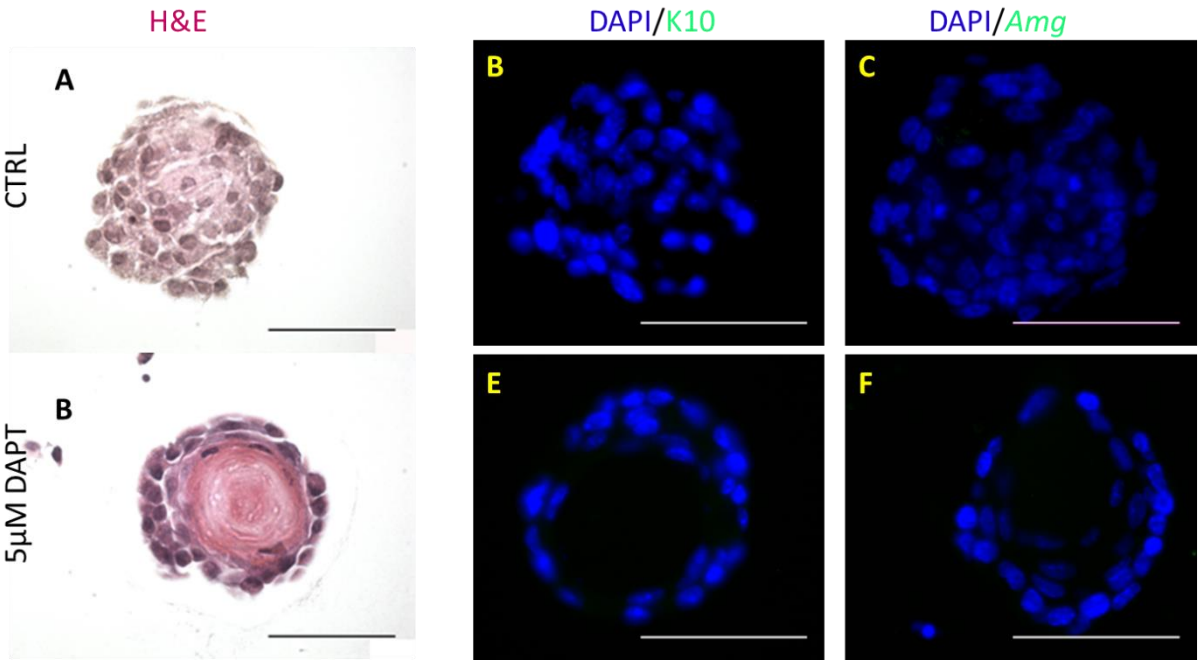


Figure 18.Effect of DAPT inhibitor. A,C) H&E staining of the forming dentospheres. Immunofluoresence against (B,E) K10 and (C,F) *Amg.* Abbreviation: DAPT: (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl ester), CTRL: Control. Scale bar:50µm.

6. 2 Lineage tracing of Notch1-expressing cells in dental epithelium.

In order to follow the progeny of Notch1-expressing cells, the reporter mice N1Cre^{ERT2} mT/mG (Fre et al. 2011) were used. Briefly, these mice express Cre recombinase under the promoter of Notch1 receptor, but that Cre should be tamoxifen-induced in order to target the floxed sequences in the Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo (gTom) transgene. Those cells in which the Cre has been activated will start expressing the Green Fluorescence Protein (GFP) and stop expressing tdTomato (Fig. 19).

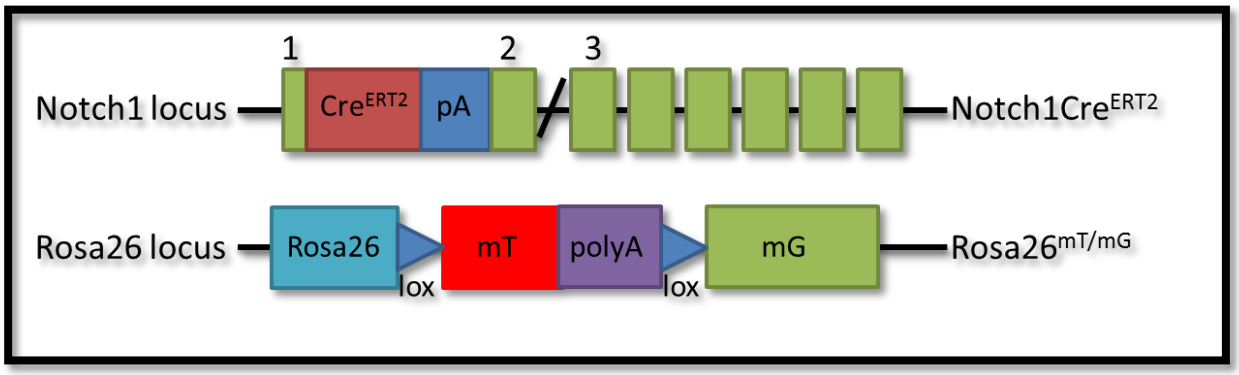


Figure 19. Schematic representation of the reporter mice N1Cre^{ERT2}mT/mG**.** Notch1Cre^{ERT2} mice were crossed with Rosa26$^{mT/mG}$ and by 4-hydrotamoxifen administration we can follow the progeny of Notch1-expressing cells since they express GFP.

6.2.1 Lineage tracing of Notch1-expressing cells during development

Incisors were dissected from E14.5 and cultured overnight with 100nM of 4-hydroxytamoxifen (4-OHT). Untreated incisors (non-induced with 4-OHT) were used as negative controls (see Appendix I).

After 1 day in culture, confocal microscopy analysis revealed the presence of Green Fluorescence Protein positive (GFP pos) cells in the middle area of the epithelium and few positive cells in the epithelial cells in contact with the dental mesenchyme in the incisors treated with 4-OHT (Fig. 20A). Interestingly, after 9 days in culture, GFP pos cells were found in cervical loop (cl), stratum intermedium (si) and stellate reticulum (sr) layers (Fig. 20B)

Analysis of other incisor 8 days after induction, GFP pos cells were observed in all the epithelial layers (Fig. 20C).

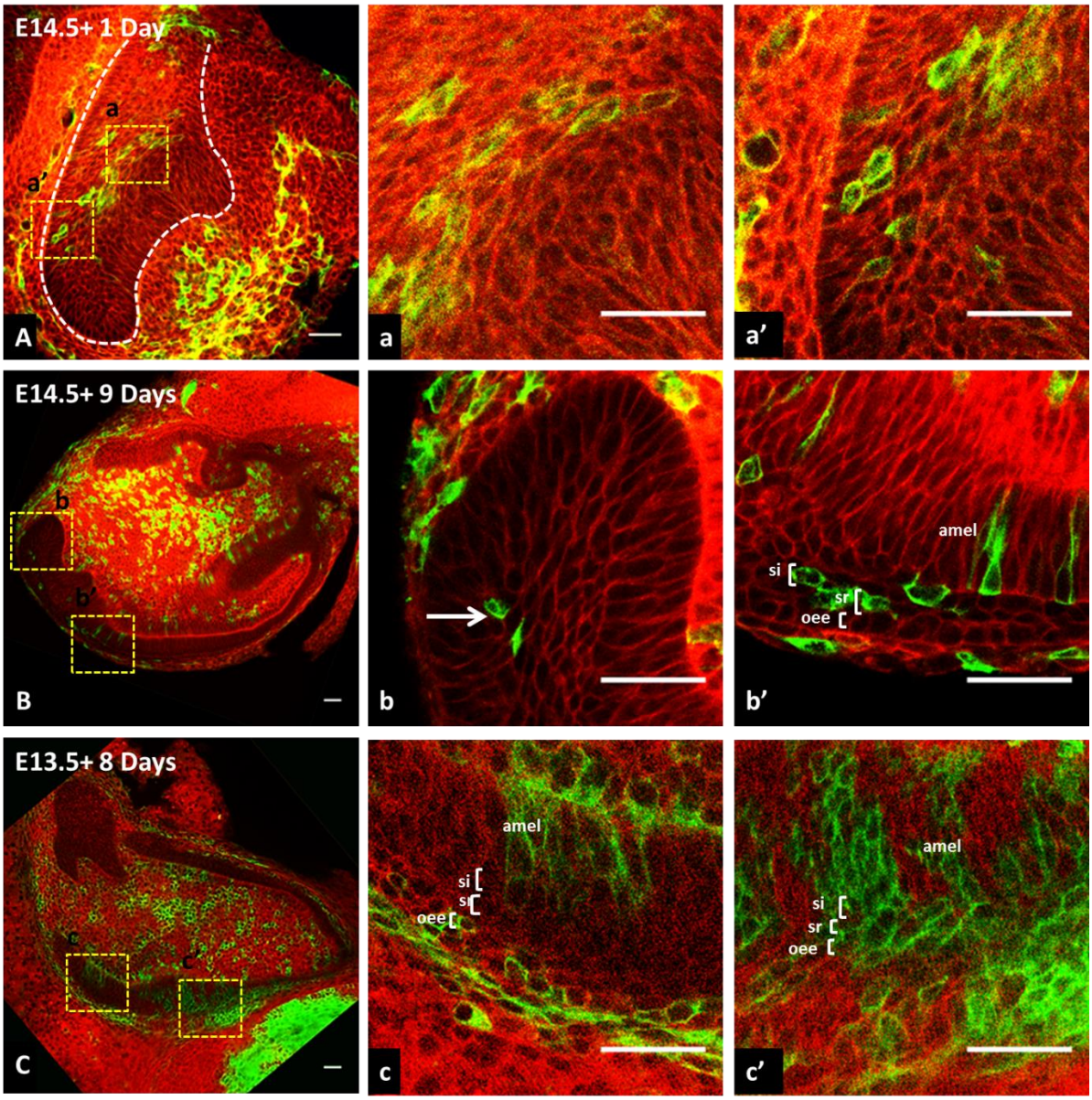


Figure 20. Lineage tracing of Notch1-expressing cells during tooth development *in vitro*. Confocal microscopy analysis of the E14.5 (A) incisor 1 day (B) 9 days and (C) 8 days after induction with 4-OHT. (a-a') In higher magnification, at day 1, GFP positive cells were found in the middle area and only few positive cells in the epithelial cells in contact with the mesenchyme. (b-b') In higher magnification, at day 9, GFP positive cells were found in cervical loop (cl), amel (ameloblasts), si (stratum intermedium) and sr (stellate reticulum). (c-c') Analysis of other incisor 8 days after induction, GFP positive cells were observed in all the epithelium lineages. Scale bar: 50µm.

6.2.2 Lineage tracing of Notch1-expressing cells in postnatal stages

In order to label Notch1 positive cells, P0 incisor were dissected and cultured overnight with 4-OHT *in vitro*. After one day, confocal microscopy analysis showed that GFP pos cells were present in cl and si, sr in the transit amplifying (TA)-Preameloblasts area (Fig. 21).

As teeth grow at a slower speed *in vitro* we performed the experiment *in vivo*. In order to label and then follow the progeny of the Notch1 positive cells *in vivo,* P3 mice were injected with a single dose of 4-OHT (Fig. 22). Incisors from mT/mG littermates (Notch1Cre negative) treated with 4-OHT did not express GFP (see Appendix I).

One day after the single injection, in the Cre positive mice, GFP pos cells were observed in the cl area, few cells in the si and sr layer in the TA-preameloblasts and secretory area of the incisor. In the maturation area GFP pos, cells were observed in the papillary layer (Fig. 23). One week after, few GFP pos cells were observed in the cl area. In the TA-preameloblasts area were observed either all the epithelial lineages GFP neg or only the sr and outer enamel epithelium (oee) GFP pos. In the secretory ameloblasts area all GFP pos cells were found in all the epithelial compartments including ameloblasts. In the maturation area, GFP pos cells were found only in the the papillary layer (Fig. 24) (Table 1).

In order to increase the labelling efficiency, P3 mice were injected two consecutive days and one day, one week and one month after were analysed (Fig. 25). One day after the second injection, GFP pos cells were observed in the cl area. In the TA-Preameloblasts area either GFP pos or GFP neg preameloblasts were observed by analysing different samples. GFP pos cells were found in the si and sr layers in all the cases. In the secretory ameloblasts si and sr were GFP pos as well as the papillary layer in the maturation area (Fig. 26). One week after the double injection, in the cl area GFP pos cells were found. In the TA-Preameloblast we observed the presence of GFP pos cells in all the epithelial compartments. However, in some samples GFP pos cells could not be detected in any epithelial layer in that area. In the secretory ameloblasts area, all the layers were containing GFP pos cells. GFP pos cells were also present in the papillary layer in the maturation area (Fig. 27). Interestingly, one month after the double injection, GFP pos cells were observed in the cl area, including the iee layer within cl. However, GFP pos cells were not observed in the area of TA-Preameloblasts. In the

maturation area, GFP pos were found in the papillary layer as well as in ameloblasts layer (Fig. 28) (Table. 2).

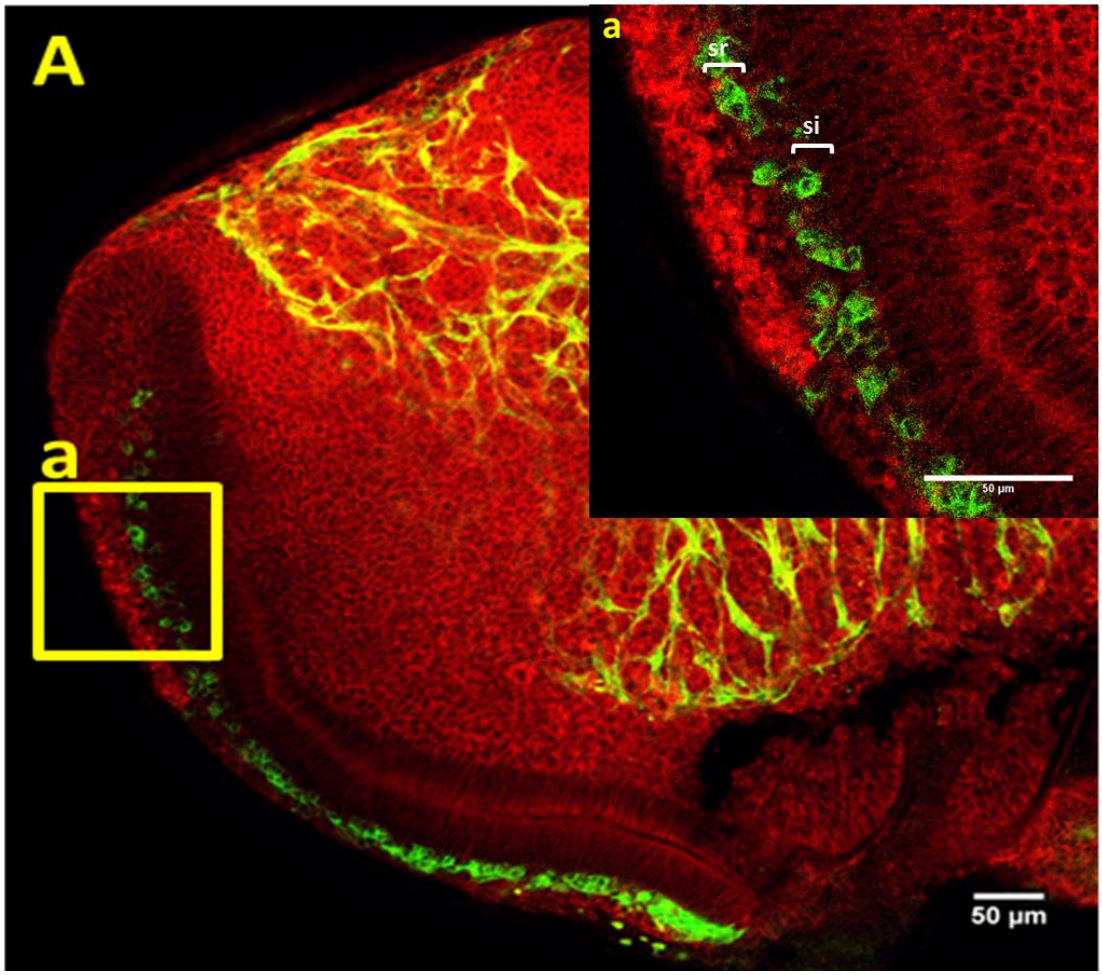


Figure 21. Confocal analysis of Notch1CreERTmT/mG incisor after 4-OHT induction *In vitro*. In green colour, GFP pos cells were observed in the (a) cl area and si/sr in the TA-Preameloblasts area. Yellow boxes represent the area of the higher magnification pictures. Abbreviation: si: Stratum intermedium, sr: Stellate reticulum.

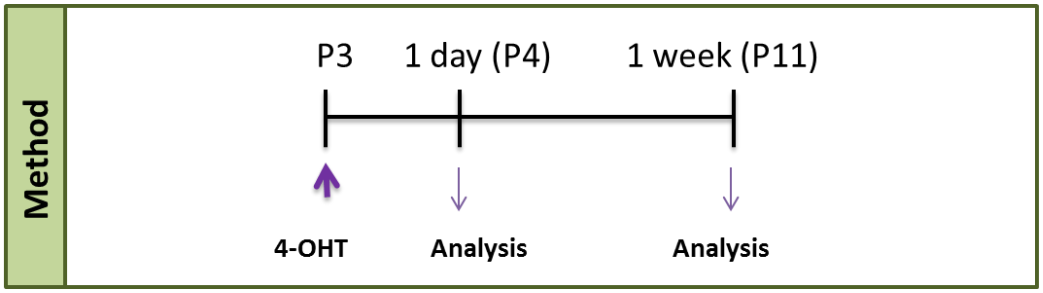


Figure 22.Timing of 4-OHT induction in Notch1CreERTmT/mG mice. P3 mice received 4-OHT intraperitoneal and were analysed 1 day and 1 week after induction.

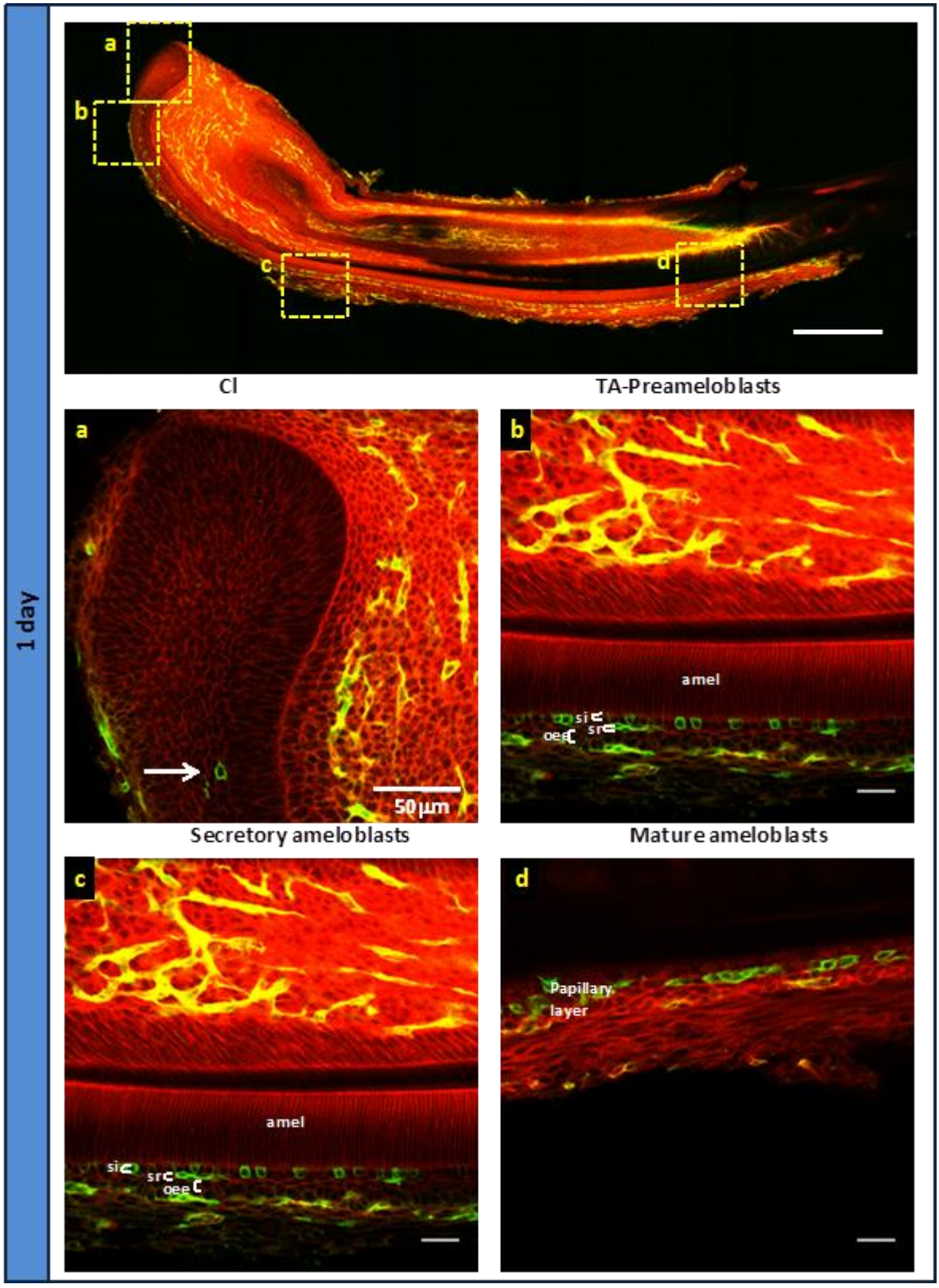

1 day
a
b
c
d
Cl
TA-Preameloblasts
a
50 µm
Secretory ameloblasts
b
amel
si
sr
oep
Mature ameloblasts
c
amel
si
sr
oep
d
Papillary
layer

Figure 23. Confocal analysis of the incisor one day after single 4-OHT injection. In green colour, GFP pos cells were observed in the (a) cl area (b) few cells in the si and sr layer in the TA-preameloblasts and (c) secretory area of the incisor as well as (d) the papillary layer in the maturation area. Yellow boxes represent the area of the higher magnification pictures. Abbreviation: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=2. Scale bar 250µm.

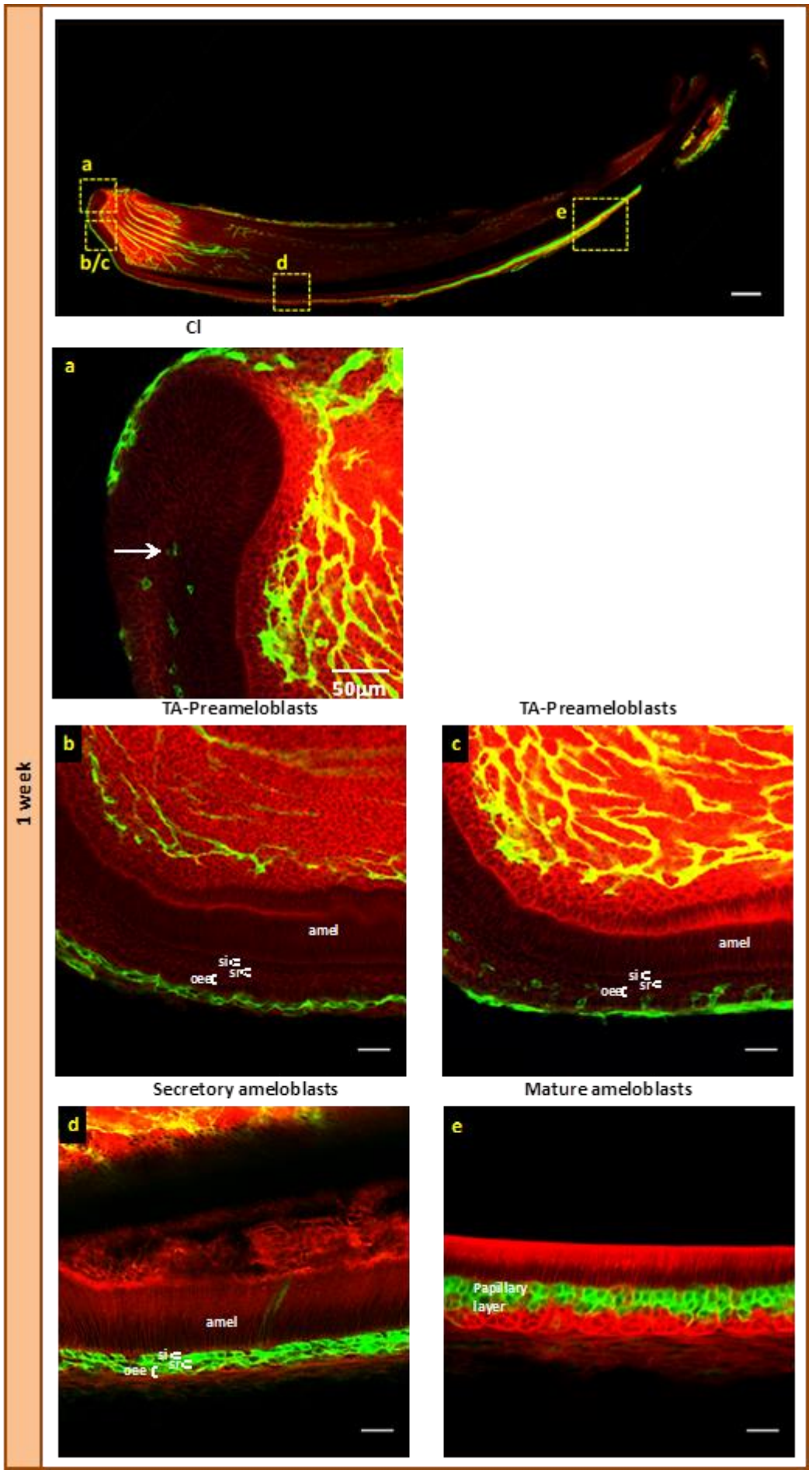

1 week
a
b/c
d
e
Cl
a
50μm
TA-Preameloblasts
TA-Preameloblasts
b
amel
oee
si
sr
Secretory ameloblasts
c
amel
oee
si
sr
Mature ameloblasts
d
amel
oee
si
sr
e
Papillary layer

Figure 24. Confocal analysis of the incisor one week after single induction. In green colour, GFP pos cells were observed in the (a) cl area (b) GFP neg cells in the TA- preameloblasts area and (c) sr and oee in the same area. (d) All the epithelial layers were GFP pos in the secretory ameloblasts area and (e) the the papillary layer in the maturation area. Abbreviation: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=2. Scale bar 250µm.

Summary

1 day

Area	Lineage	Notch1
CL		+
TA/Preameloblasts	iee	-
	si	+
	sr	+
	oee	-
Secretory ameloblasts	amel	-
	si	+
	sr	+
	oee	-
Mature ameloblasts	amel	-
	papilary	+

1 week

Area	Lineage	Notch1
CL		+
TA/Preameloblasts	iee	-
	si	-
	sr	+/-
	oee	+/-
Secretory ameloblasts	amel	+
	si	+
	sr	+
	oee	-
Mature ameloblasts	amel	-
	papilary	+

Table 1. Summary of the observation of the GFP pos cells in the different epithelial layers after single 4-OHT injection. In the summary box, + represents that GFP pos cells were detected within that layer whereas – means that no GFP pos were observed. +/- means that in some analysed incisors GFP pos cells were observed within that layer whereas in other incisors, no GFP pos cells could be found in such compartment. Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum.

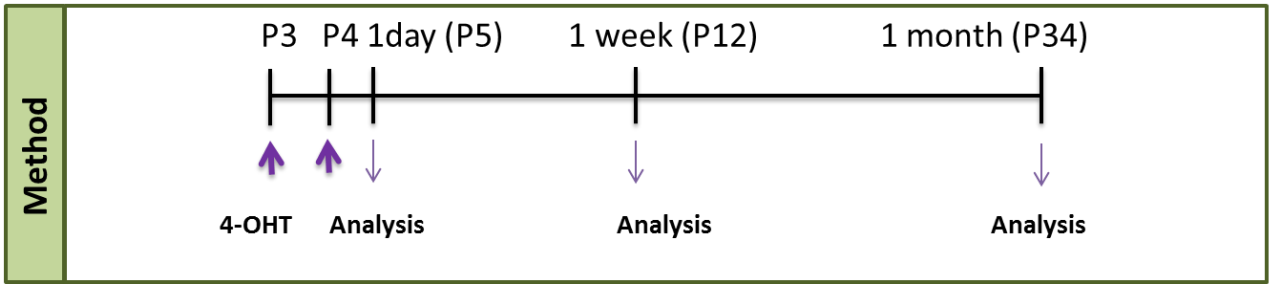


Figure 25.Timing of 4-oht double induction in Notch1CreERTmT/mG mice. P3 mice received two consecutive days 4-OHT intraperitoneal and were analysed 1 day and 1 week and 1 month after induction.

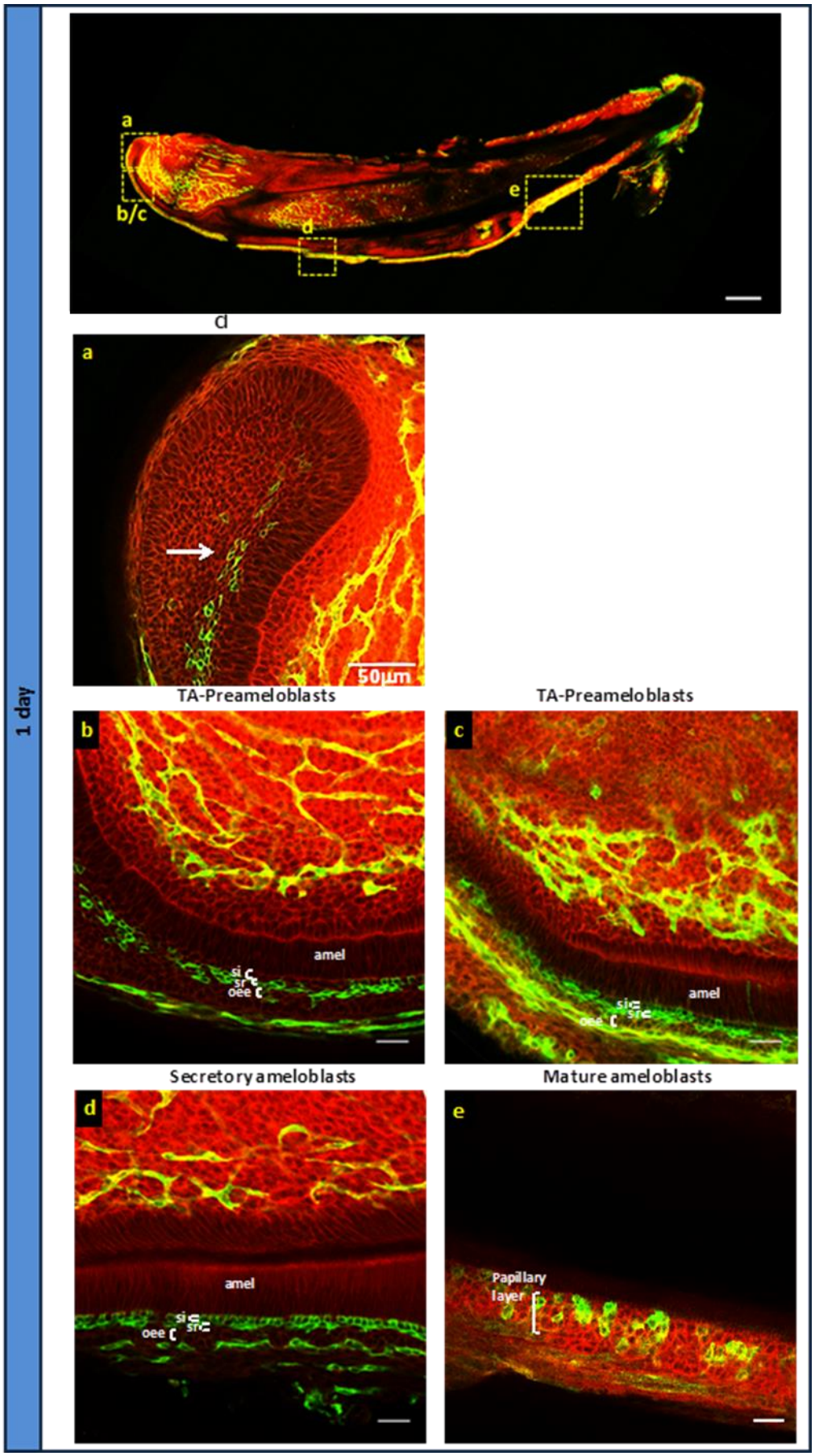

1 day
a
b/c
d
e
d
a
50µm
TA-Preameloblasts
TA-Preameloblasts
b
amel
si
sr
oee
c
amel
si
sr
oee
Secretory ameloblasts
Mature ameloblasts
d
amel
si
sr
oee
e
Papillary layer

Figure 26. Confocal analysis of the incisor one day after double 4-OHT injection. In green colour, GFP pos cells were observed in (a) the cl area, (b) few cells in the si and sr layer in the TA/preameloblasts as well as (c) preameloblasts in same case, (d) si and sr layers in secretory area and (e) the papillary layer in the maturation area. Yellow boxes represent the area of the higher magnification pictures. Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=3. Scale bar 250µm

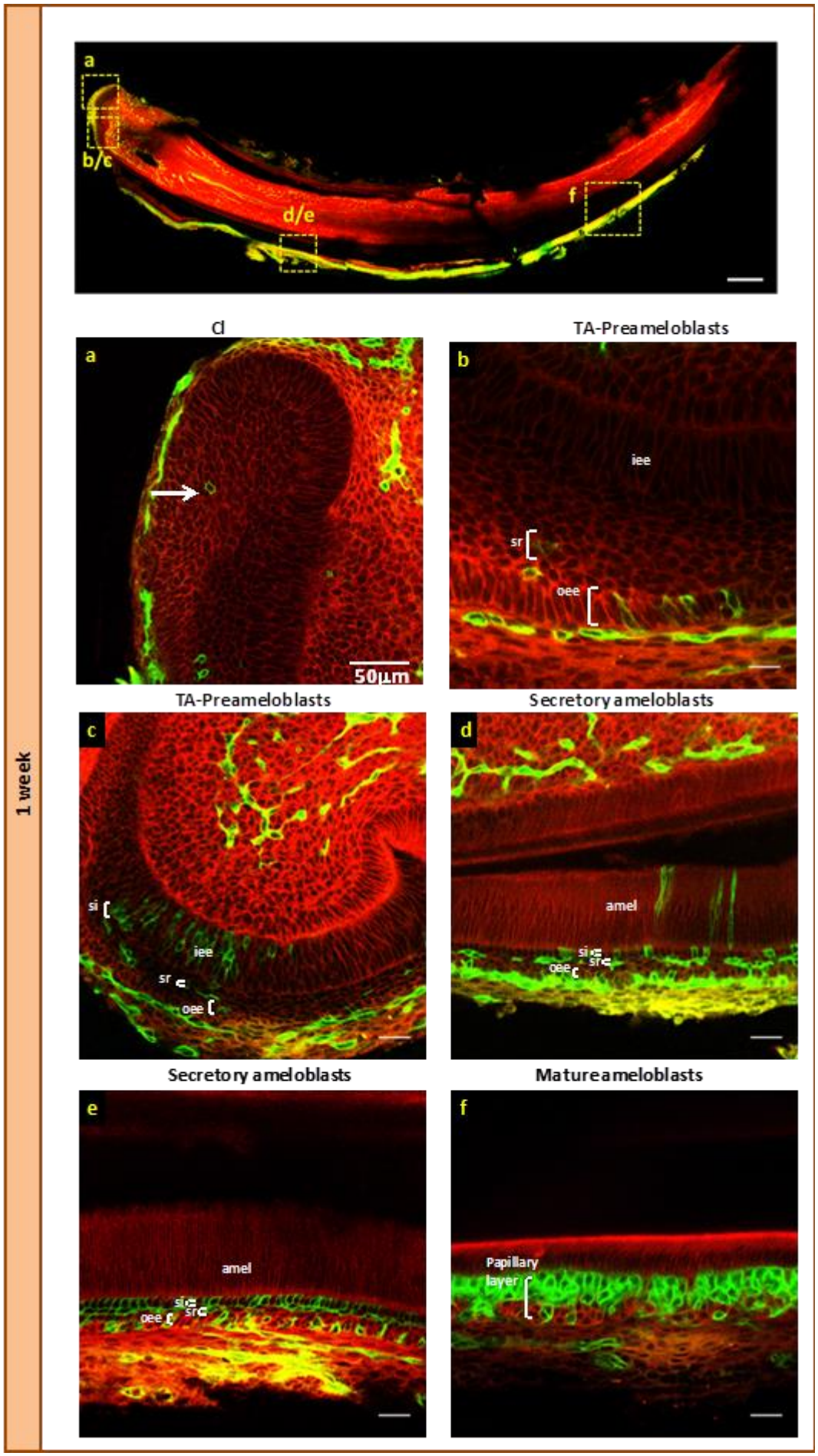

1 week
a
b/c
d/e
f
Cl
TA-Preameloblasts
a
b
iee
sr
oee
50µm
TA-Preameloblasts
Secretory ameloblasts
c
d
si
iee
sr
oee
amel
si
sr
oee
Secretory ameloblasts
Mature ameloblasts
e
f
amel
si
sr
oee
Papillary layer

Figure 27. Confocal analysis of the incisor one week after double induction. In green colour, GFP pos cells were observed in the (a) cl (b) GFP neg cells in the TA- preameloblasts area and (c) sr and oee in the same area. (d) All the epithelial layers were GFP pos in the secretory ameloblasts area and (e) the the papillary layer in the maturation area. Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=3. Scale bar 250µm.

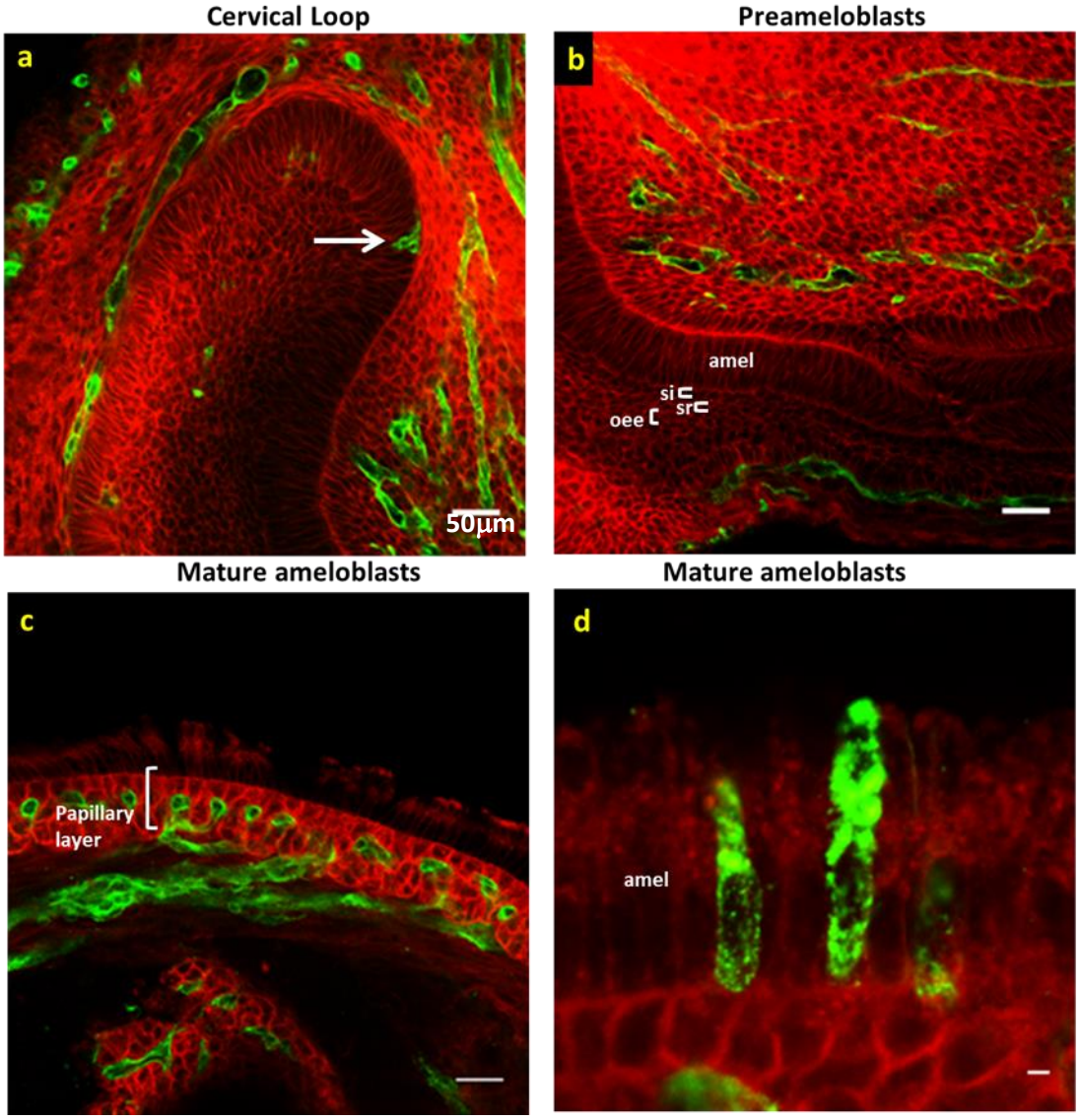


Figure 28. Confocal analysis of the incisor one month after double induction. In green colour, GFP pos cells were observed in cl (a) not in the TA Preameloblasts area (b) and in the papillary layer (c) as well as ameloblasts (d) in the maturation area.Abbreviation: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=2, Scale bar: 250µm.

Summary

1 day

Area	Lineage	Notch1
CL		+
TA-Preameloblasts	iee	-/+
	si	+
	sr	+
	oee	-
Secretory ameloblasts	amel	-
	si	+
	sr	+
	oee	-
Mature ameloblasts	amel	-
	papilary	+

1 week

Area	Lineage	Notch1
CL		+
TA-Preameloblasts	iee	+/-
	si	+/-
	sr	+/-
	oee	+/-
Secretory ameloblasts	amel	+/-
	si	+
	sr	+
	oee	+
Mature ameloblasts	amel	-
	papilary	+

1 month

Area	Lineage	Notch1
CL		+
TA-Preameloblasts	iee	-
	si	-
	sr	-
	oee	-
Mature ameloblasts	amel	+
	papilary	+

Table 2. Summary of the observation of the GFP pos cells after double injection. In the summary box with + shown the GFP positive cells, with – shown the negative GFP cells and +/- both Positive/negative GFP cells were observed. Abbreviation: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum.

6.2.3 Lineage tracing of Notch1-expressing cells during regeneration

In order to assess the potential of Notch1-expressing cells during regeneration following injury three different ways were used. Firstly, an injury in the cl area were the stem/progenitors cells reside was performed. P3 incisors were dissected and after Cre activation with 4-OHT *in vitro*, an injury was performed in the edge of the cl in some of the incisors. Some other incisors were left intact and used as controls. After 5 days *in vitro*, by confocal analysis few more GFP pos cells were observed in the area where the injury was performed (Fig. 29) when compared to the control incisors.

Then we used an *in vivo* approach in order to cause injury in incisors. Thus, we aimed to activate the regeneration capacity of the incisor by trimming it. After three consecutive 4-OHT injections to increase the labeling efficiency, incisors of postnatal mice were trimmed in the erupted part. Completely regrowth of incisors was observed after 7 days. As a control uninjured incisors were used. We couldn't observe any difference between injured and control incisors (Fig. 30).

In order to perform more severe injury, one day after double 4-OHT injection in P3 mice, the anterior part of the incisor was cut off and the posterior part containing the cl area was transplanted under the kidney capsule of immunocompromised mice. Ten days after, the samples were analysed by confocal analysis. We observed that the injured incisor presented a high number of GFP- pos ameloblasts while in intact incisors they rarely contribute to the generation of this cell type. We repeated the same injury experiment with incisors from mice in which Cre was activated by double injection one month before. Similar to the previous experiment in which incisors were injured one day after the Cre activation, we observe a significant number of GFP pos ameloblast in the injured incisor, while very rare GFP pos cells in the ameloblasts layer were find in the control (Fig. 31).

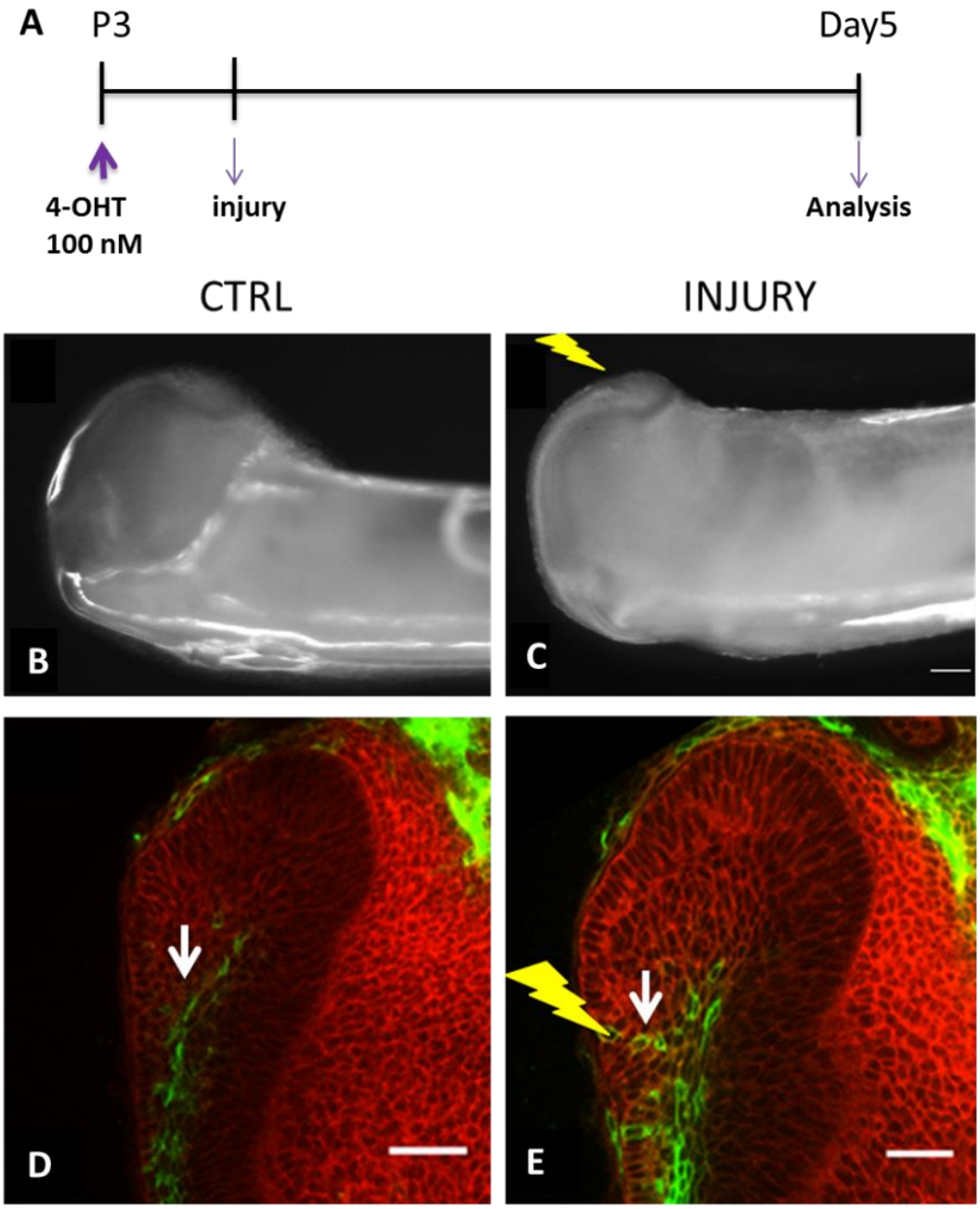


Figure 29. Injury in the cl area in vitro. As a control the intact incisor were used (A) Injury was performed in the edge of the cl (B).In green colour, more Notch1-expressing cells were observed in the cl area where the cl was injured(C-D).n=3. Scale bar: 50µm

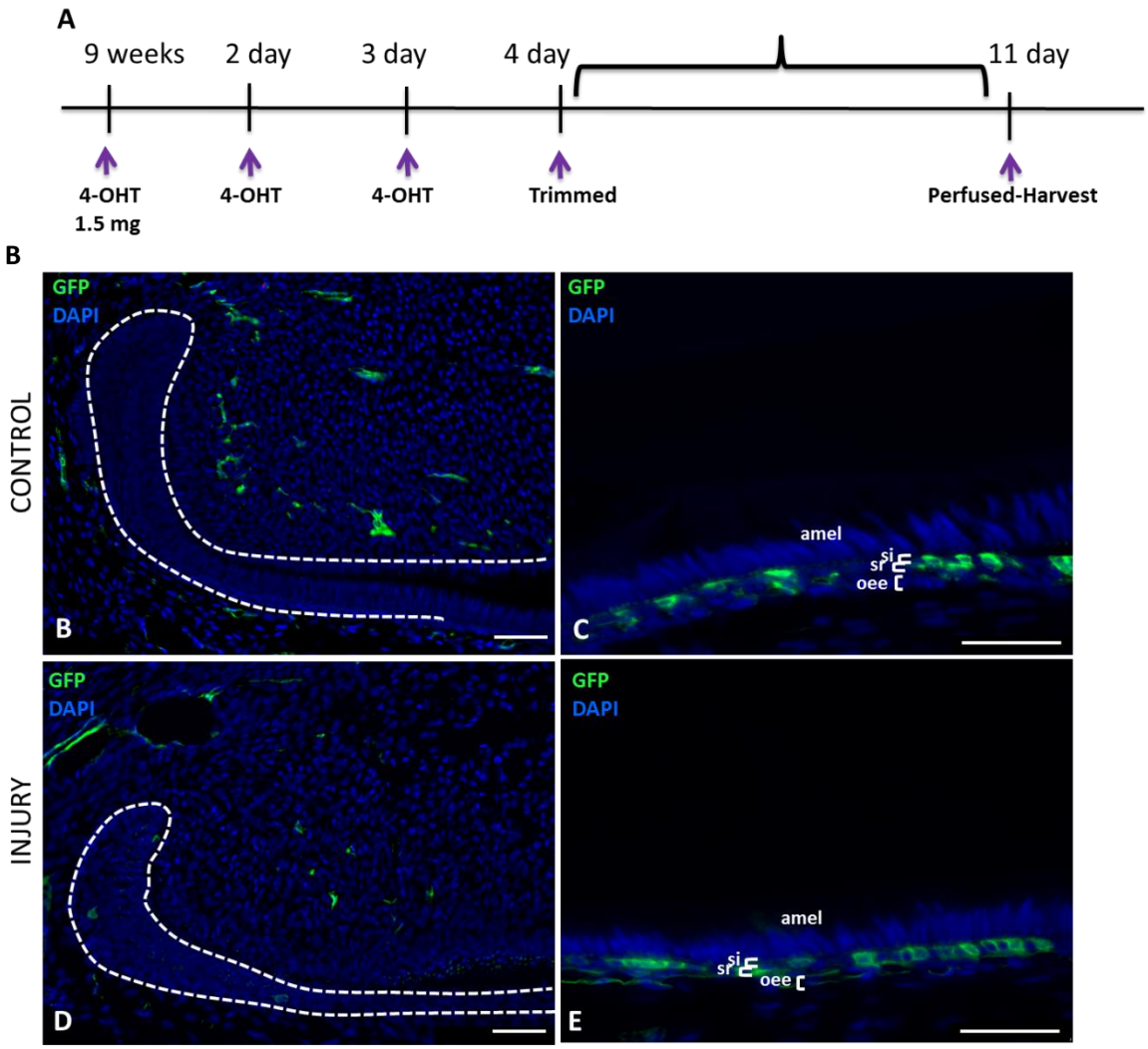


Figure 30. Trimming the adult incisor. A) Timeline of the experiment. B) Immunofluoresce against GFP to detect the progeny of Notch1 expressing cells in intact control (B-C) and trimmed incisors (D-E). Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=3. Scale bar: 50µm

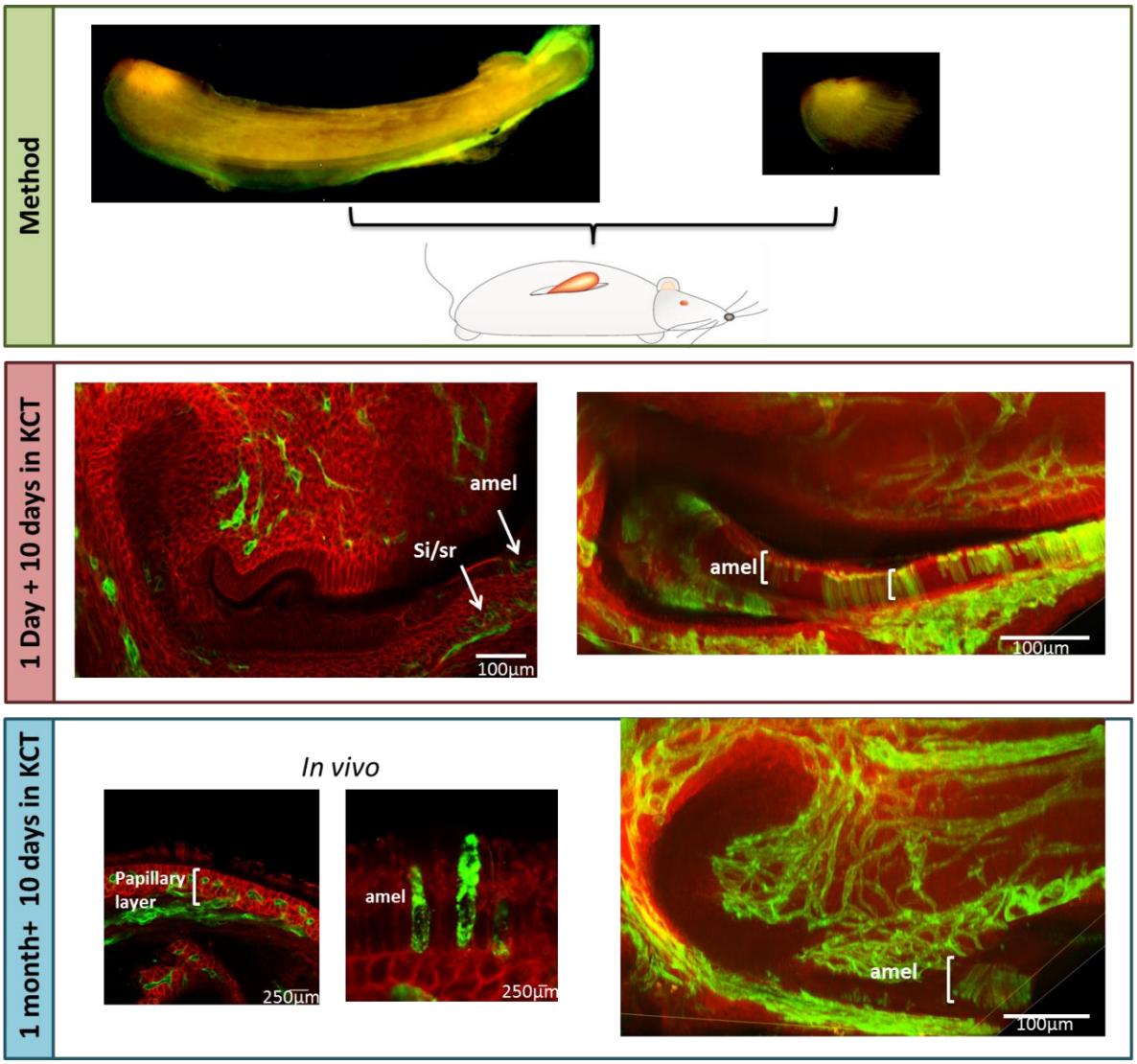


Figure 31. Incisor injury model for the study of the plasticity of Notch1-expressing cells during regeneration following damage. Whole intact incisors and cut posterior parts were transplanted under the kidney capsule of immunocompromised mice. High numbers of GFP pos ameloblasts (green colour) were observed in incisors injured one day after 4-OHT double injection while in intact incisors they rarely contribute to the generation of this cell type. One month after cre activation, only rare GFP pos cells were observed in the ameloblasts layer in the maturation area. However, when the incisors are injured one month after double 4-OHT injection, the contribution of Notch1-expressing cells to the formation of ameloblasts increased. n=2. Abbreviations: amel: ameloblasts, KCT: Kidney Capsule Transplantation.

6.3 Role of Notch1 in cell fate determination of DESCs *in vivo*.

The role of Notch1 in cell fate determination of DESCs postnatally and after injury repair process were analysed by using the transgenic mouse K14CreNotch1$^{fl/fl}$ (referred as K14N1cKO) in a 129S2/SvPasCrl background. Briefly, Notch1$^{flox/flox}$ mice were crossed with K14Cre mice in order to analyse the effect of the loss of Notch1 function in dental epithelium (Fig. 32).

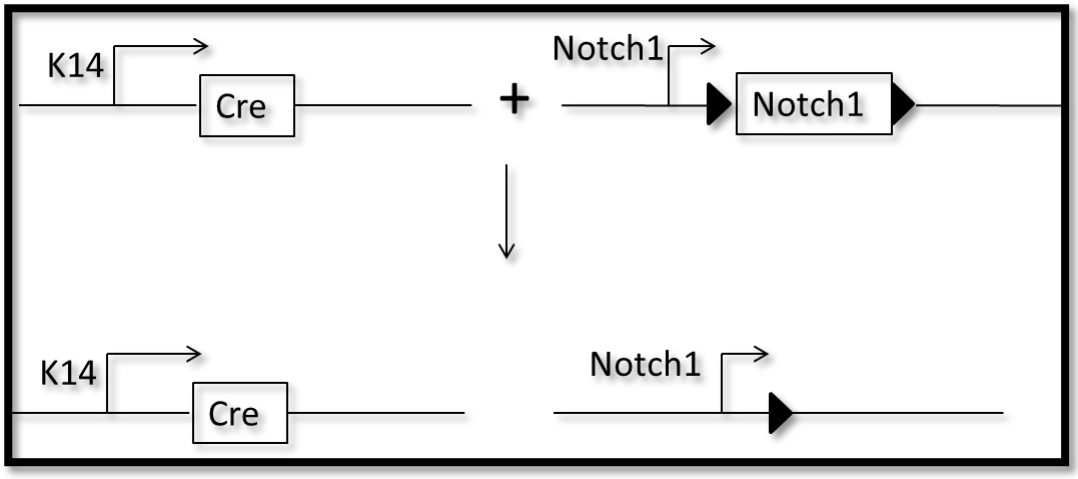


Figure 32. Schematic representation of the transgenic mouse line K14N1cKO. K14Cre mice were crossed with Notch1$^{flox/flox}$ mice in order to specifically delete Nocth1 expression in the dental epithelium.

6.3.1 Role of Notch1 in cell fate determination of DESCs postnatally

Macroscopically the enamel was found to be discoloured in K14N1cKO when compared ro wildtype mice, either suggesting a problem in the mineralization, or thickening or composition of the enamel (Fig. 33A,C). However, SEM did not reveal any severe defect in the thickening or mineralization of the enamel (Fig. 33). Higher magnification pictures did not show any defect in the organization of the basic structural units of the enamel known as enamel rods (Fig. 33b,d). We could not observe any obvious alteration in the distribution of iron in the K14N1 KO upper and lower incisors by prussian blue staining (Fig. 34). Further histological analysis by H&E staining did not show any significant alteration in any of the different dental epithelial cell types (Fig. 35). In addition, we did not detect significant changes in the expression pattern of ameloblasts markers *Amg* and *Amb* by *in situ* hybridization (Fig. 36).

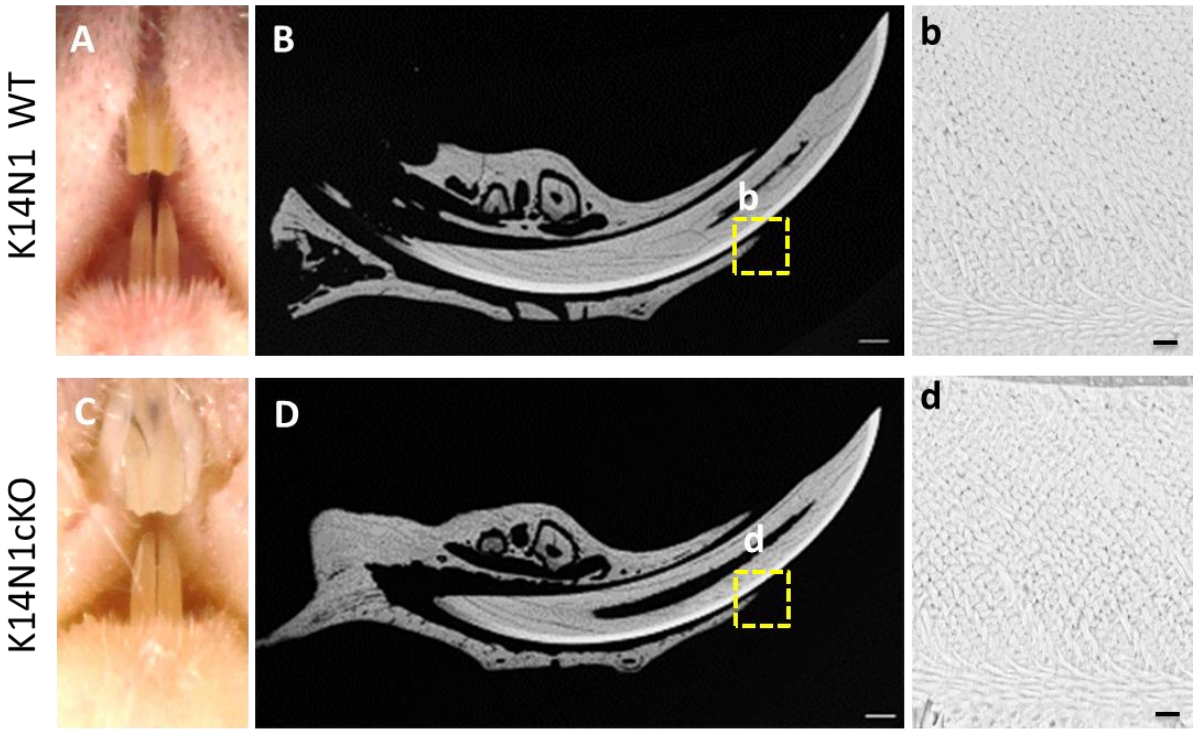


Figure 33. Comparison of K14N1WT and K14N1 KO postnatal mice. Macroscopical analysis showing discoloured enamel in mutant (C) vs control (A) mice. SEM analysis didn't show any dramatic change neither in the structure (B-D) nor in the mineralization of the enamel (b-d). n=3. Scale bars: 50 µm (B-D), 5 µm (b-d).

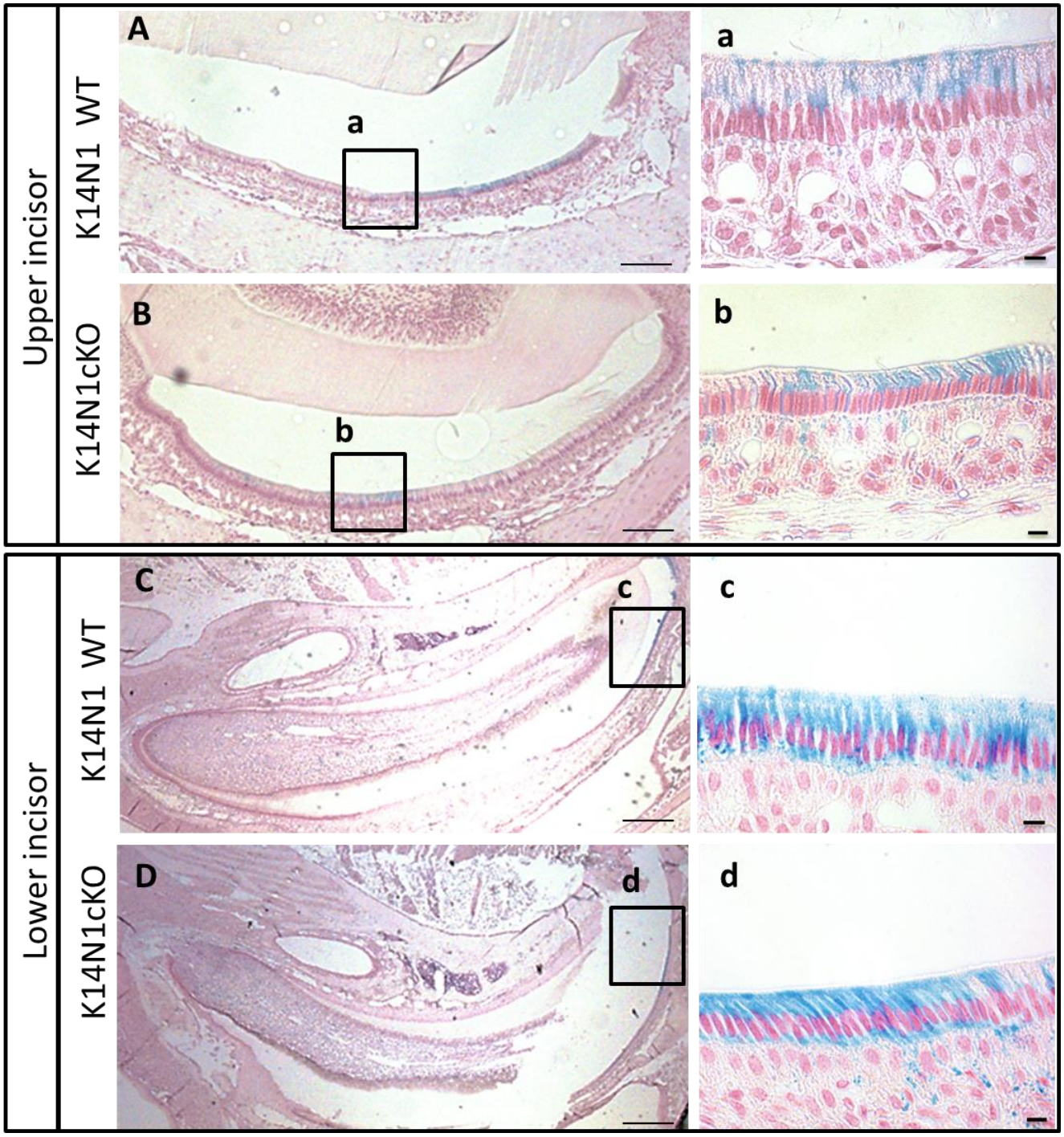


Figure 34. Prussian blue staining in paraffin sections of K14N1WT and K14N1cKO incisors. The presence and distribution of iron was similar in incisors from K14N1WT and K14N1 KO mice, in both the upper (A-B) and lower (C-D) incisors. Higher-magnification views of the boxed regions for upper incisor (a-b) and lower incisor (c-d). n=3. Scale bar: A-B:100 µm, C-D: 500 µm, a-d: 10 µm.

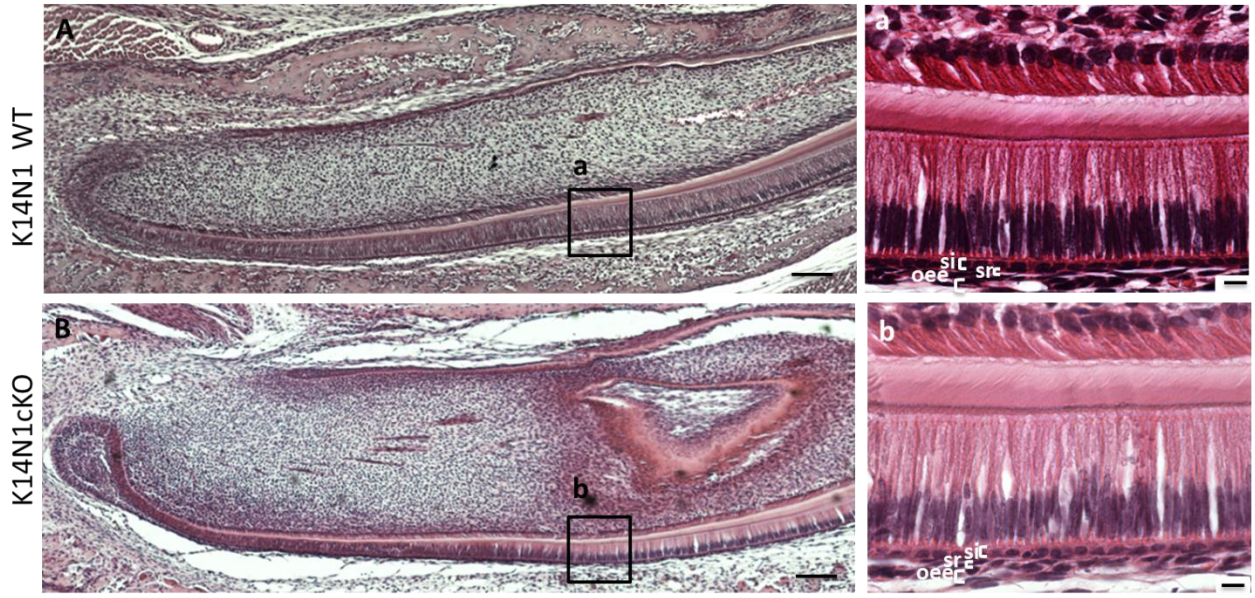


Figure 35. Histological analysis of K14N1WT and K14N1cKO incisors. H&E staining did not show any difference in the epithelium between K14N1WT and K14N1 KO incisors. Higher-magnifications of the boxed regions (a, b). Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=3. Scale bar: (A-B):100 µm, (a-b): 10 µm.

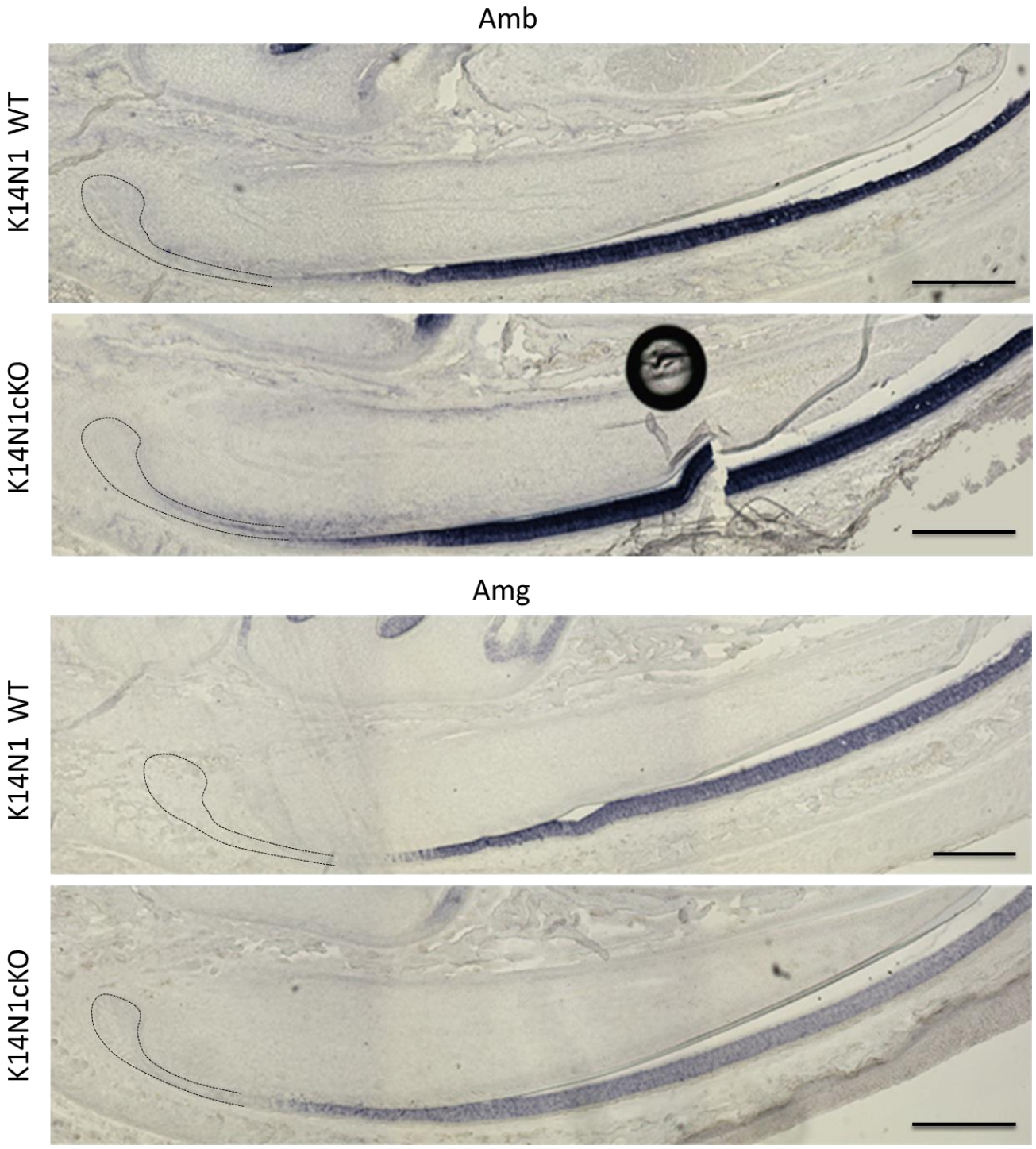


Figure 36. In situ hybridization of K14N1WT and K14N1cKO incisor. The expression of *Amb* and *Amg* was similar in K14N1WT and K14N1 KO incisors. Scale bar: 250µm.

6.3.2 Role of Notch1 in cell fate determination of DESCs during regeneration

Mouse incisors grow continuously throughout the life of the animal. In order to observe possible stronger phenotype of the deletion of Notch1, injury repair assay was performed. Briefly, the anterior part of the K14N1cKO incisors was cut off and the posterior part containing the cl area was transplanted under the kidney capsule of immunocompromised mice. The intact incisors were used as a control. Ten days after, the samples were analysed by H&E staining. We did not observe any striking difference between injured and intact K14N1cKO dental epithelium (Fig. 37).

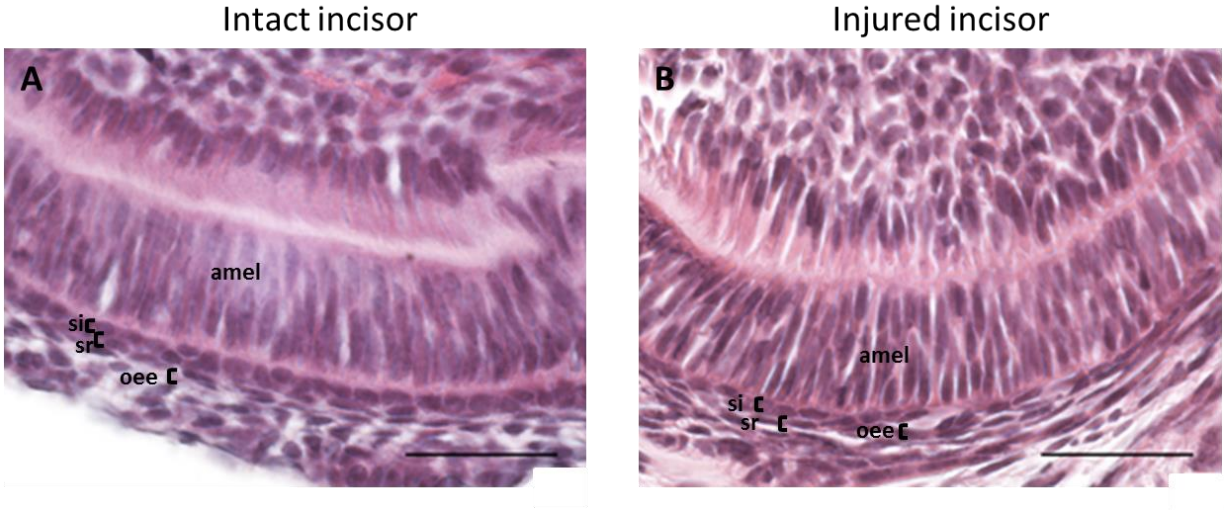


Figure 37. H&E staining of intact and injured K14N1cKO incisors. (A) The intact and (B) injured incisor. No differences in the epithelium were observed. Abbreviations: amel: ameloblasts, Iee: inner enamel epithelium, oee: outer enamel epithelium, si: Stratum intermedium, sr: Stellate reticulum. n=3. Scale bar 50µm.

6.3.3 Analysis of the efficiency of the deletion

As we did not observe any obvious phenotype in K14N1cKO mice, we checked the efficiency of the Notch1 deletion by backcrossing the K14N1cKO mice with the Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J (mT/mG) reporter mice. Using this reporter system, those cells in which Cre is activated should also express GFP. Confocal analysis of incisors from P1 K14N1 WT mice revealed the presence of several GFP neg cells in all the different areas of dental epithelium

including cervical loop. GFP neg cells were found in all dental epithelial cell compartments (Fig. 38).

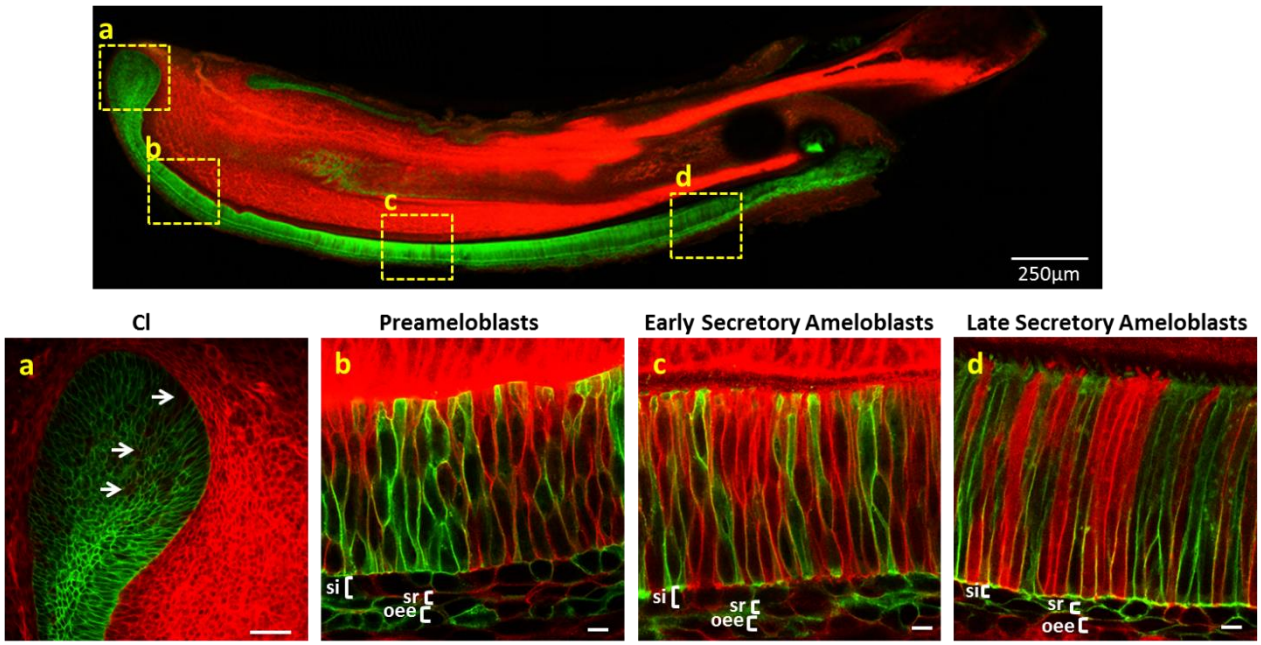


Figure 38. Confocal analysis of K14CreNotch1$^{wt/wt}$ mT/mG incisors. GFP negative cells were observed in the epithelium in all the different areas and cell compartments. Higher-magnification views of the boxed regions. Abbreviations: amel: ameloblasts, si: Stratum intermedium, sr: Stellate reticulum, oee: outer enamel epithelium. n=3. Scale bars 25µm.

7. Discussion

7.1 Ability of DESCs to form spheres

Based on the capacity of stem cells from other tissues to form spheres *in vitro*, we aimed to optimize the culture conditions for dental epithelial stem cells (DESCs) to grow as spheres and characterize them (Seaberg et al., 2004; Shackleton et al., 2006; Chiasson et al., 1999; Lombaert et al., 2008; Kawase et al., 2004; Dontu et al., 2003; Chen et al., 2005). During the development of this project, it has been shown that DESCs isolated from the cervical loop (cl) of the mouse incisor have the ability to grow as spheres when cultured *in vitro* (Chavez et al., 2013; Chang et al., 2013; Chang et al., 2013b). However, little was reported about the identity and stem cell properties of the formed spheres.

Initially, we used different combinations of growth factors known to be involved in important biological processes. For example, fibroblast growth factors (FGFs) control many important cell processes like survival, proliferation and differentiation. FGFs are characterized in the literature as very important stem cell regulators (Jiménez-Rojo et al., 2012; Harada et al., 1999). FGF10 has been found to play a role in stem cell maintenance in the incisor cl (Harada et al., 2002) and FGF2 is of crucial importance for the maintenance of human embryonic stem cell self-renewal (Parsa et al., 2010; Diecke et al., 2008). Also, it has been shown that FGF signalling is preventing DESC differentiation in an *in vitro* sphere assay (Chang et al., 2013). Epidermal growth factor (EGF) is also associated with important cell functions such as survival, proliferation and differentiation (Howell 2004). Initially, we compared different combinations of growth factors and we observed that cells treated with FGF2/EGF can form spheres of bigger size (25μm in diameter on average) and with higher efficiency (10 spheres per 10.000 cells) when compared to other factors. However, when compared to other spheres like the mammospheres and salispheres, our spheres were still very small and the efficiency was low (Dontu et al., 2003). Thus, we decided to embed single dental epithelial stem cells in Matrigel and expose them to the same combinations of growth factors as in the previous experiment plus a new medium consisting on keratinocyte serum-free medium (KSFM) supplemented with human recombinant EGF and bovine pituitary extract (EGF/BPE). Under all these conditions, sphere formation was observed, with higher efficiency and bigger diameter than the cells cultured without Matrigel.

Phase contrast microscopy and hematoxylin & eosin (H&E) staining of sphere sections in all conditions revealed DESCs sphere morphology. Under all the conditions except for the cells treated with KSFM supplemented with EGF/BPE, the spheres presented a skin epithelium -like structure with different layers and a keratinized center. Thus, these results suggest that the spheres were differentiating into non-dental structures. In contrast, cells treated with KSFM and EGF/BPE were forming round mass-like structured dentospheres containing only dental epithelial stem cells. The efficiency of dental stem cells to form spheres was higher and the size of the spheres was similar to those derived from epithelial tissues of other organs (Seaberg et al., 2004; Shackleton et al., 2006; Chiasson et al. 1999; Lombaert et al. 2008; Kawase et al., 2004; Dontu et al., 2003; Chen et al., 2005)

7.2 Inhibition of Notch during formation of epithelial dentospheres

After establishing the optimal conditions to expand DESCs and form dentospheres, we aimed to investigate the role of Notch in sphere formation. We cultured DESC spheres in the presence or absence of Notch inhibitor, (N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl ester) (DAPT), at a final concentration of 5 μM for 10 days. Interestingly we observed by phase contrast microscopy and H&E staining round mass-like structured dentospheres in the presence of Notch inhibitor. However, inside the spheres exposed to the Notch inhibitor, an extracellular matrix-like structure was observed. Although we were not able to identify the nature of that matrix (it did not express the epidermal marker K10 or the enamel matrix marker Amg), its presence suggest that inhibiton of Notch signaling leads to the differentiation of DESCs.

Additionally, the efficiency to form dentospheres was lower in the presence of the Notch inhibitor when compared to the control. This suggests that Notch signaling may be crucial for the proliferation and survival of DESCs and goes in line with previous data showing the requirement of Notch signalling for epithelial stem cell survival (Felszeghy et al., 2010).

7.3 Notch1 pos epithelial cells are multipotent stem cells during embryonic incisor development

To examine the lineage potential of Notch1 expressing epithelial cells during embryonic incisor development, we labelled the Notch1 pos cells by 4-hydroxytamoxifen (4-OHT) induction *in vitro* in dissected E14.5 incisors. One day after induction, we found Notch1 expressing cells mainly located in the inner mass area corresponding to the area where the stratum intermedium (si) and stellate reticulum (sr) are located in the later stages. Additionally, very few Notch1 pos cells were observed in the outer layers of the epithelium. However, Notch1 is normally expressed in the inner mass of the E14.5 incisor (Mitsiadis et al., 1998). Thus, our results suggest that already 24 hours after the induction few progeny cells were also labelled, indicating that it may be necessary to analyze the incisors at earlier time points (between 12-20 hours) to ensure that we are detecting only Notch1-expressing cells and not the progeny.

After 9 days, we observed Notch1 pos cells in all the epithelial components. Together, these results demonstrate that Notch1 pos cells are multipotent stem cells during development, which has also been observed in other ectodermal organs like mammary glands (Rodilla et al., 2015).

7.4 Notch1 pos epithelial cells are multipotent stem cells in postnatal stages

To determine whether the Notch1 pos epithelial cells are indeed stem cells that can give rise to all the different epithelial lineages in the postnatal mouse incisor, the progeny of genetically labelled Notch1 expressing epithelial cells was first traced *in vitro.* In P0 dissected incisors, we detected Notch1 pos cells in the cl area as well as in si/sr layers after overnight induction with 4-OHT. These results fit with the expression of *Notch1* observed by *in situ* hybridization (Natsiou, unpublished data).

Next, we aimed to follow the progeny of the Notch1 pos dental epithelial cells *in vivo*. Initially, we analyzed the location of Notch1 pos cells after one day of a single dose of 4-OHT induction and we observed that Notch1 pos cells were located in the cl as well as in both si and sr layers in all the different areas of the incisor, as expected.

In another set of experiments, we performed double injections of 4-OHT in two consecutive days and we observed the presence of more GFP positive cells along dental epithelium, suggesting that indeed we could target more cells. Higher labeling efficiency by double 4-OHT induction was detected also in the case of Sox2CreERT mice (Juuri et al., 2012).

Thus, when we increased the labeling efficiency by double injections, Notch1 pos cells were also observed in other cell layers such as preameloblasts in the transient amplifying (TA)-preameloblasts area. This could be due to the increased labeling efficiency of Notch1 pos cells after double injection. As we know from *in situ* hybridization data, *Notch1* is not expressed in preameloblasts at that stage (Natsiou, unpublished data). Therefore, the fact that we observed Notch1 pos preameloblasts in the TA-preameloblast area after 48 hours of 4-OHT induction suggests that we were able to target the progeny already.

Subsequently, to follow the progeny of the Notch1 pos cells, we analyzed the incisor one week after the 4-OHT injection. In the case of single 4-OHT injections, Notch1 expressing cells were labeled in the cl area. In the TA-preameloblasts area we observed GFP expressing cells in both si and outer enamel epithelium (oee) layers. After double 4-OHT injection, we observed Notch1 pos cells in the cl area and there were cases that we saw Notch1 pos cells only in sr and oee, while in most of the cases Notch1 pos cells were observed in all the layers. These results suggest that there is a continuous generation of epithelial cells from Notch1-expressing cells located in the cervical loop area.

One month later, after double 4-OHT injection, Notch1 pos cells were still found in the cl and Notch1 pos mature ameloblasts were also observed. Ameloblasts in the mouse incisor are known to advance around 350 μm per day (Hwang and Tonna, 1965). Therefore, these mature ameloblasts are most probably generated from Notch1 expressing cells in cl.

The fact that we could observe Notch1 pos cells in the cl area at every time point suggests that Notch1 pos cells in postnatal incisor may be stem cells. Our data shows that Notch1-expressing cells are continously generating cells that contribute to all the different dental epithelial compartments. This indicates that Notch1 pos cells in postnatal incisors were able to generate all the four epithelial lineages suggesting that Notch1 expressing cells are multipotent stem cells. Similarly, by lineage tracing experiments, it has been observed that Sox2, Bmi1 and Gli1 positive cells are stem cells (Juuri et al., 2012; Seidel et al., 2010; Biehs et

al., 2014; Zhao et al., 2014). Interestingly, Notch1-expressing cells are located in a well-defined territory within the cervical loop that differs from the locations of other previously identified stem cell populations (Fig. 39) suggesting that they constitute a new stem cell population in dental epithelium of postnatal incisor.

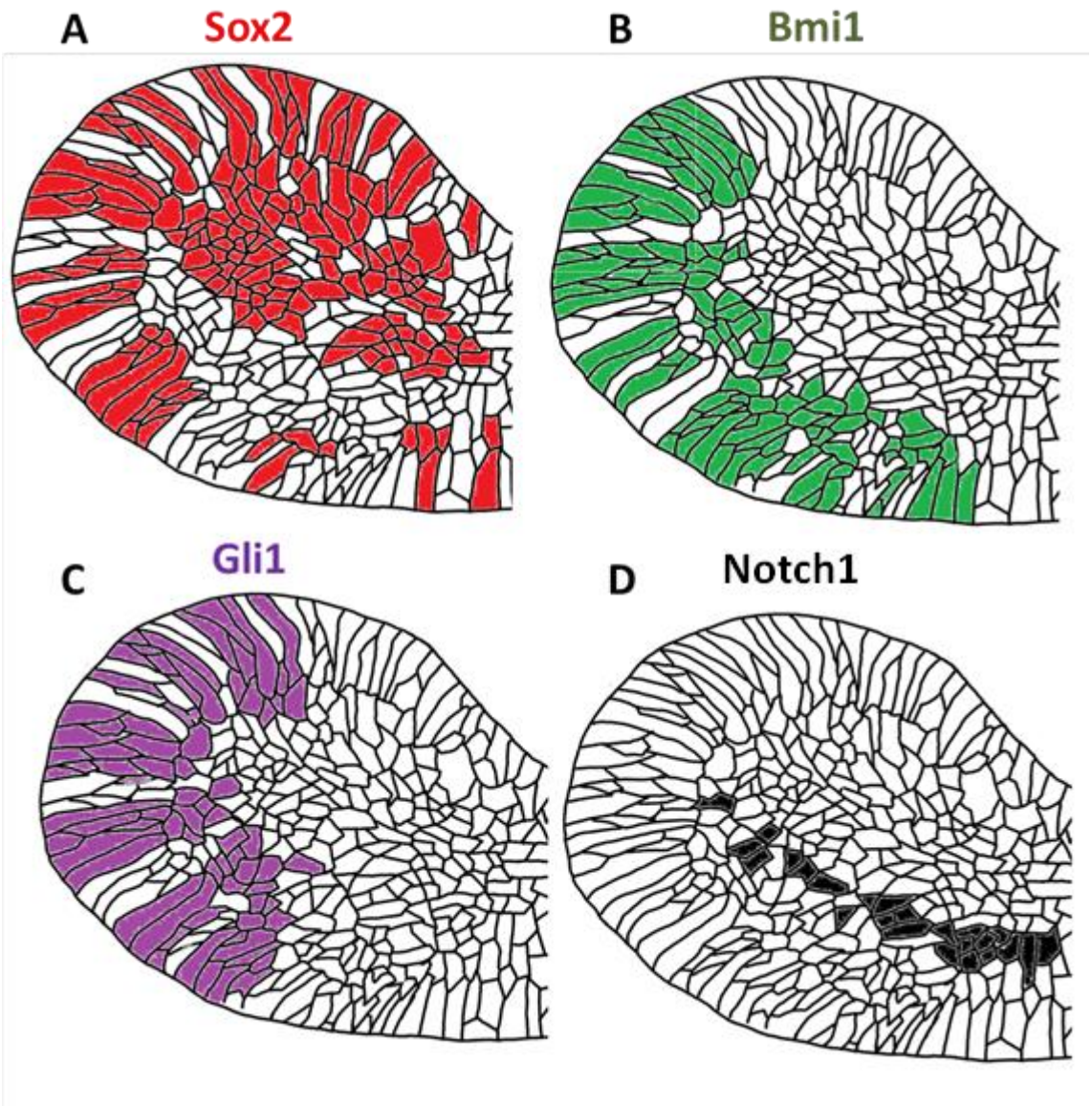


Figure 39. Schematic representation of the different populations of stem cells within the cl area of the mouse incisor. (A) Sox2, (B) Bmi1, (C) Gli1 and (D) Notch1.

7.5 Notch1 pos epithelial cells are activated upon injury

As previouly mentioned, different DESC populations have been identified so far in postnatal incisor. However, there is no information about their behaviour during regeneration after injury.

In order to test the fate of the Notch1 pos dental epithelial cells during regeneration following injury we used three different approaches. Initially, we made an injury in the cl area were the stem/progenitors reside and we observed after five days few more Notch1 pos cells in the area where the injury was performed. However, the difference was not striking, probably because the injury was not severe enough.

Thus, we tried to perform a more severe damage by trimming the erupted part of the adult incisor (after administrating 4-OHT for three consecutive days). However, we observed the same amount of Notch1 pos cells as in the control, suggesting that the injury was not severe enough and/or that the Notch1 pos cells were not targeted efficiently enough (only few Notch1 efficiently cells were observed in the cl).

As these two previous approaches did not show any significant changes, we tried to create a more severe injury by cutting the posterior part of postnatal incisors containing the cl area and letting it grow under the kidney capsule of immunocompromised mice. Here, high numbers of Notch1 pos ameloblasts were observed, while in intact incisors they rarely contributed to the generation of these cells. Similarly, when the injury was induced one month after 4-OHT induction, an increased number of Notch1 pos ameloblasts when was observed when compared to control incisor. These results suggest that Notch1 expressing cells highly contribute to the formation of enamel-forming ameloblasts after injury.

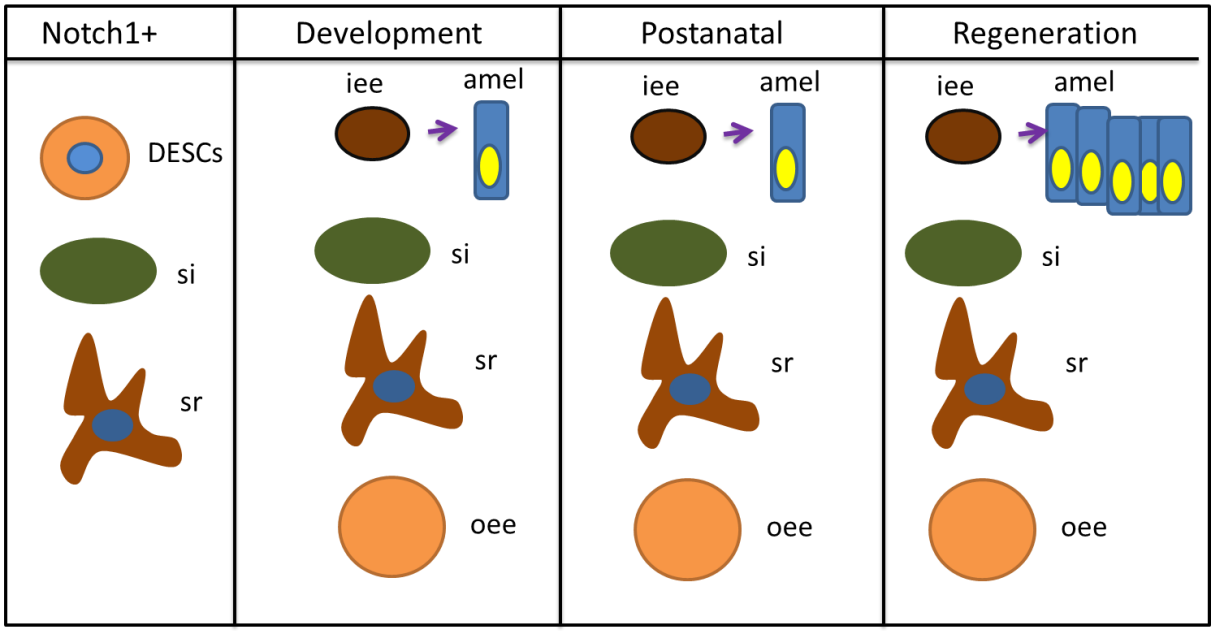


Figure 40. Summary of the fate of the Notch1 pos epithelial cells. Notch1 epithelial cells are multipotent stem cells during development and homeostasis with higher ability to generate ameloblasts during injury-repair processes.

7. 6 Inefficient deletion of Notch1 in K14N1cKO mice

The role of signalling pathways, including the Bmp, Fgf, Shh, and Wnt pathways, during tooth development as well as during renewal has been well characterized (Jheon et al., 2013). However, the role of Notch signalling during tooth development and renewal has not been studied as thoroughly as the other major pathways. Recently, one study has shown a minor defect in the epithelial lineages like partial flattening of the si layer in the apical-basal direction by injecting a blocking antibody against Notch1 (Jheon et al., 2015). However, by injecting the Notch1 inhibitor the effect on DESCs might not be specific but caused by a more general effect. Instead, using transgenic mice could be a better way to analyse the role of Notch1 in cell fate determination of DEPCs. Therefore, we analyzed K14N1cKO mice, in which Notch1 expression is expected to be defective in the dental epithelium. Macroscopically, the enamel was found to be discolored either suggesting a problem in the mineralization, or in the thickening or composition of the enamel. Enamel is mainly composed of minerals (>90%) (Barlett, 2013), therefore, we used scanning electron microscopy (SEM) to analyze the incisors of 2 months old mice. Our results did not reveal any severe defect in the mineralization of the enamel, which showed spatially normal ordered prisms. Additionally, we could not observe

any evident reduction in the enamel thickness of the knock-out (KO) compared to the wild-type (WT). Using Prussian blue staining we visualized the iron location and we found the composition of the enamel to be normal. As we could not observe any defect neither of the mineralization nor thickening nor composition of the enamel, we further analyzed the different epithelial cell layers of postnatal incisors. However, histological analysis did not show any significant alteration in dental epithelium of K14N1cKO mice. Additionally, no significant defect was observed in the expression pattern of different proteins including Ameloblastin (*Amb)* and Amelogenin (*Amg)*. Generally, these proteins show significant alterations in more severe genetic disorders of enamel, generally termed Amelogenesis Imperfecta (Gibson et al., 2001).

Over the past few decades, Notch signaling has been shown to play a fundamental role not only during development and homeostasis but also after injury repair (Koch et al., 2013; Vauclair et al., 2007). For example, in the cornea, stem/progenitor cells within the corneal epithelium participate in wound repair, while Notch1-deficient stem/progenitor cells form a keratinized plaque (Vauclair et al., 2007). However, in our results we could not observe any abnormality in the dental epithelium after injury repair assay.

By using conditional animals, embryonic lethality can be bypassed and cell lineages for whose development a given target gene is critical can be identified. However, the Cre loxp system also have limitations. The fact that we could not observe any severe phenotype suggested a general problem with the Cre/loxp system. Thus, we checked the efficiency of the Notch1 deletion by backcrossing the K14N1 mice with the Gt(ROSA)26Sortm4 (ACTB-tdTomato,-EGFP)Luo/J reporter mice. Indeed, we observed a high number of cells in which Cre recombinase was not active in all epithelial layers showing that the Cre was not efficiently targeting all the dental epithelial cells.

7. 7 Conclusions and perspectives

In this study, we analysed the role of Notch signalling in cell fate determination of dental epithelial stem/progenitor cells. After establishing optimal conditions to expand DESCs from the cl area of the incisor, we showed that Notch is required for the proliferation and survival

of DESCs and is preventing DESC differentiation. By using *in vitro* and *in vivo* lineage-tracing assays we have showed that Notch1-expressing cells are multipotent stem cells during embryonic development and in postnatal stages of the incisor, with higher ability to generate ameloblasts after injury repair. However, since with K14N1cKO mice we could not efficiently delete Notch1 from dental epithelium, we need to use a different strategy (such as using a different Cre driver mouse line) in order to unravel the role of Notch1 in dental epithelium *in vivo*.

In summary, we have identified a new population of Notch1-expressing dental epithelial stem cells that are increase their ability to generate enamel-producing ameloblasts in a severely injured postnatal incisor.

Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
ADAM	tumor necrosis factor α-converting enzyme
Amb	Ameloblastin
Amg	Amelogenin
BPE	Bovine Pituitary Extract
Brdu	5-Bromo-2′-deoxyuridine
CS	Calf serum
Cl	Cervical loop
CoR	co-repressor
CSL	Suppressor of Hairless and Lag-1
DAPT	N-[N-(3,5-Difluorophenylacetyl-L-alanyl)]-S-phenylglycine t-butyl este)
DESCs	Dental epithelial stem cells
DiI	1,1′-dioctadecyl-6,6-di(4-sulfophenyl)-3,3,3′,3′-tetramethylindocarbocyanine
Dig1	Drosophila disc large tumor suppressor
Dll1	Delta like 1
DMEM	Dulbecco's Modified Eagle Medium
DSL	Delta Serrate homologues
EDTA	ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Epfn	Epiprofin/Sp6
H&E	Hematoxylin/Eosin
E10.5/14.5/18.5	Embryonic stage 10.5/14.5/18.5
FACS	fluorescence activated sorting
FGF	Fibroblast growth factor
HBSS	Hanks' Balanced Salt Solution
HDACS	proteins and histone deacetylases
HD	and heterodimerization domain
Hh	Hedgehog
H2BGFP	histone 2B-Green Fluorescence Protein
Iee	Inner enamel epithelium

KCT	Kidney capsule transplantation
KO	Knock-out
KSFM	Keratinocyte serum-free medium
Lgr5	G-protein coupled receptor
MAML1-3	Mastermind-like protein
NCT	transmemrane proteins nicasterin
NECD	Notch extracellular domain
Next	Notch extracellular truncation
NICD	Notch intracellular domain
Notch1 pos	Notch1 positive
Notch1 neg	Notch1 negative
LN	cysteine-rich Notch/Lin12 domains
Oee	Outer enamel epithelium
PBS	Phosphate buffer saline
PDZ	post synaptic density protein
PEST	serine and threonine-rich motifs sequence
PFA	Paraformaldehyde
SEM	Scanning electron microscopy
SHH	Sonic hedgehog
Si	Stratum intermedium
S	Site 1
SKIP	SKI-interaction protein
Sr	stallate reticulum
SRY	sex determining region Y
TA	transit amplifying
TAD	transactivation domain
TMD	single transmembrane domain
YAP	Yes-associated protein
WT	Wild-type
Wnt	vertebrate homologue of the Drosophila Wingless gene
3D	3 Dimensional
4-OHT	4-Hydroxytamoxifen

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First of all, I would like to thank my supervisor and mentor Prof. Thimios Mitsiadis, for giving me the opportunity and support to carry out my PhD in his lab. Especially, thanks to my direct-supervisor Dr. Lucia Jimenez-Rojo since without her support and input it wouldn't be possible to perform this research.

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Most of all I would like to thank my mother, sister, grandparents and boyfriend for their constant love, care, and support during these years.

10. Curriculum Vitae

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Permit	B Permit with eligibility to work in Switzerland
Marital status	Single

EDUCATION AND QUALIFICATION

2013 – Jan 2017	**PhD at Molecular Life Science Graduate School,** **ETH/University of Zürich, Switzerland** PhD Title: Role of Notch signalling in cell fate determination of dental epithelial stem/progenitor cells
2012 – 2013	**Master of Research in Biomedical Science** **Beatson Institute for cancer research, University of Glasgow, United Kingdom** Project 1: Cytokeratins, Fibroblast growth factors in the mouse urothelium of genetically engineered mice **Institute of Oral Biology, Center of Dental Medicine, University of Zürich** Project 2: Orofacial Development and Regeneration, Three-dimensional in vitro culture for the expansion of dental epithelial stem cells
2008 – 2011	**Bachelor of Science in Microbiology and Biotechnology** **University of Portsmouth, United Kingdom** Project: Epigenetic Modifications and their role in cancer
2007-2008	**Assessment and Qualifications** **Highbury College Portsmouth, United Kingdom** Alliance for entry to Higher Educations

SKILLS AND COMPETENCES

Languages	Greek	Mother tongue
	English	Fluent, oral and written
	German	Good, oral and written
	French	Good, oral and written
Computer skills	MS-Office	Excellent Knowledge
	Photoshop	Advanced Knowledge
	Image J	Advanced Knowledge
	SPSS	Basic Knowledge

WORK EXPERIENCE

University of Portsmouth, United Kingdom, Research Associate

- Worked as part of a research group to determine the cytotoxicity of biomaterial as well as the use of FTIR (ATR technique) for the characterisation of polymeric films deposited on dental surfaces.
- Responsibilities include carrying out relevant lab protocols, following health and safety procedures, maintaining a safe and hygienic environment in the lab and supporting the research team.

TRAINING SCHOOLS/CONFERENCES

- Central European Initiate, Practical course and workshop: Application of Neural Stem Cells and Mouse Models in Neuroscience, 02-05 October 20013, 3D in vitro culture for the expansion of dental epithelial stem cells. D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (oral presentation)
- Training School Cost Action NAMABIO MP1005 3rd Course, From nano to macro biomaterials and application to stem cells regenerative orthopedic and dental medicine, Zagreb, Croatia, 26-29 April 2014, 3D in vitro culture for the expansion of dental epithelial stem cells. D. Natsiou, T. Mitsiadis, L.Jimenez-Rojo (oral presentation)
- 10th Annual Swiss Stem Cell Network Meeting, SSCN, Geneva, Switzerland, 4 June 2014, Characterization of dental epithelial stem cells from mouse teeth. D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (poster)
- 13th Day of Clinical Research, University of Zurich, Switzerland, 12 June 2014, 3D in vitro culture for the expansion of dental epithelial stem cells. D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (poster)
- 11th International Conference on Nanosciences & Nanotechnologies, Thessaloniki, Greece, 08-11 July 2014), 3D in vitro culture for the expansion of dental epithelial stem cells. D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (oral presentation)
- 11th Annual Retreat of MLS,Rigi Kaltbad, Switzerland, 28-30 August 14, Role of Notch1 signaling pathway in the regeneration of dental epithelial stem cells. D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (poster)
- 12th Annual Retreat of MLS,Engelberg, Switzerland, 27-31 August 15, Lineage tracing of Notch1 cells in dental epithelium during tooth development and homeostasis.D. Natsiou, T. Mitsiadis, L. Jimenez-Rojo (poster)

TEACHING

- Teaching assistant: Classical and Molecular Genetics – BIO11 (Bachelor of Science UZH in Biology), Module leader: Prof. Michael Hengartner, (November-December 2014), (October - December 2015)
- Teaching assistant: Classical and Molecular Genetics – BIO17 (Bachelor of Science UZH in Biology), Module leader: Prof. Basler, (October –January 2015)

SCHOLARSHIPS

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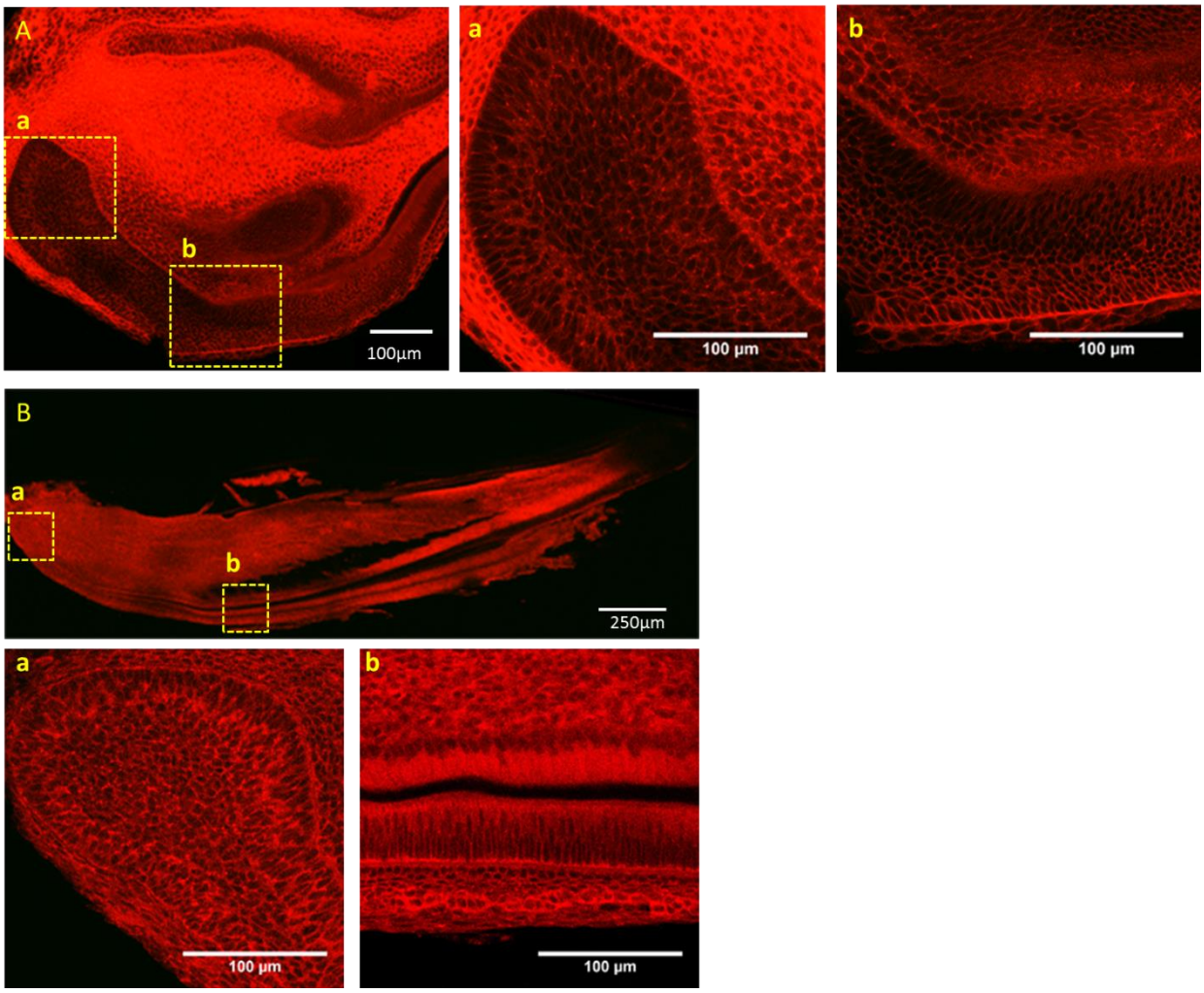
- 2014: COST NAMABIO Training School

PUBLICATION

Orsini, G., Jimenez-Rojo, L., **Natsiou, D.,** Putignano, A., & Mitsiadis, T. a. (2015). In vivo administration of dental epithelial stem cells at the apical end of the mouse incisor. Frontiers in Physiology, 6 (April).

Natsiou, D., Granchi, Z., Mitsiadis, T. a., Jimenez-Rojo, L. (2017). Generation of Spheres from Dental Epithelial Stem Cells, 19 (January).

11. Appendix I



Confocal analysis of incisors from negative controls used to validate N1CreERTmT/mG mice. A) N1CreERTmT/mG incisors not treated with 4-OHT. B) mT/mG incisors (N1CreERT negative) one day after 4-OHT treatment.

12. Appendix II

frontiers in Physiology

METHODS
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In vivo administration of dental epithelial stem cells at the apical end of the mouse incisor

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Cell-based tissue regeneration is an attractive approach that complements traditional surgical techniques for replacement of injured and lost tissues. The continuously growing rodent incisor provides an excellent model system for investigating cellular and molecular mechanisms that underlie tooth renewal and regeneration. An active population of dental epithelial progenitor/stem cells located at the posterior part of the incisor, commonly called cervical loop area, ensures the continuous supply of cells that are responsible for the secretion of enamel matrix. To explore the potential of these epithelial cells in therapeutic approaches dealing with enamel defects, we have developed a new method for their *in vivo* administration in the posterior part of the incisor. Here, we provide the step-by-step protocol for the isolation of dental epithelial stem cells and their delivery at targeted areas of the jaw. This simple and yet powerful protocol, consisting in drilling a hole in the mandibular bone, in close proximity to the cervical loop area of the incisor, followed up by injection of stem cells, is feasible, reliable, and effective. This *in vivo* approach opens new horizons and possibilities for cellular therapies involving pathological and injured dental tissues.

Keywords: mouse incisor, enamel, tooth, dental injury, dental pathology, regeneration, stem cells, cervical loop

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Introduction

The continuously erupting rodent incisor represents a suitable model system for studying cell proliferation, migration, differentiation, and mineral matrix deposition during development, homeostasis and regeneration of organs. Two of the hardest tissues of the body, the enamel and dentin, form as the outcome of interactions between oral epithelium cells and the cranial neural crest-derived mesenchyme during odontogenesis (Mitsiadis and Graf, 2009; Mitsiadis and Luder, 2011). The mineralized dental tissues are vulnerable to various external harmful agents, and to traumatic injuries that jeopardize tooth integrity. Loss of dental hard tissues in rodents caused by the frequent chewing and gnawing is balanced by constant cell divisions at the apical end of the incisor, allowing thus *de novo* enamel and dentin matrix formation by newly differentiated cells. Indeed, *in vivo* and *in vitro* cell tracing studies have shown that the cervical loops, which are located at the posterior part of the incisor, are niches for dental epithelial stem cells (DESCs) (Harada et al., 1999; Mitsiadis et al., 2007; Mitsiadis and Graf, 2009; Li et al., 2012). It has been demonstrated that DESCs are able to give rise to all epithelial cell layers of the incisor, including the enamel-forming layer of ameloblasts (Juuri et al., 2012; Biehs et al., 2013). Despite the obvious differences between rodent

incisors and human teeth that include morphological, physiological and functional criteria there are fundamental similarities in dental hard tissue formation and structure in most of the species (Warshawsky et al., 1981; Jheon et al., 2013). However, damaged enamel cannot be repaired naturally in human teeth since ameloblasts are not present anymore after tooth eruption. Therefore, dental stem cells combined with tissue engineering products could be useful for the development of innovative strategies for cell-based dental tissue regeneration in the clinics (Mitsiadis et al., 2012).

To investigate the potential of DESCs in dental tissue regeneration and repair, we have applied an experimental model consisting of drilling a "window" in the alveolar bone of the mouse mandible, which overlies the apical part of the incisor. The creation of this bone window allows the injection of the DESCs at precise areas of the jaw, without affecting the overall physiology and masticatory attitudes of the animal. Here we demonstrate that this technique is successful and can be efficiently used to *in vivo* administer DESCs that could eventually be used for the repair of damaged or pathological dental tissues.

Materials and Methods

Isolation of Dental Epithelial Stems Cells

I. Dissect incisors from postnatal day 7 (PN7) ROSA26-EGFP mice. Incubate the incisors for 20 min at RT in Dispase (2 mg/ml) and DNAse (20 U/ml) solution in HBSS. Separate mechanically the epithelium from mesenchyme and dissect the cervical loop area.
II. Add the tissues in 15 ml Falcon tubes with 14 ml of PBS/10% CS.
III. Centrifuge at 300 g for 5 min.
IV. Remove supernatant.
V. Add 1 ml of PBS.
VI. Centrifuge at 300 g for 5 min.
VII. Remove supernatant.
VIII. Add 200 μl of 0.25% Trypsin (in PBS) and incubate 30 min at 37°C.
IX. Mix gently and pipet up and down vigorously.
X. Add DNase I (2 U/ml) and incubate 5 min at 37°C.
XI. Add 700 μl of PBS/10% CS.
XII. Centrifuge at 300 g for 5 min.
XIII. Remove supernatant.
XIV. Add 1 ml of PBS.
XV. Centrifuge at 300 g for 5 min.
XVI. Remove supernatant and resuspend DESCs in DMEM/F12 medium (1 ml).
XVII. Filter the cells through 40 μm cell strainer.
XVIII. Count the cells.
XIX. Pellet the cells at 300 g for 5 min.
XX. Resuspend DESCs in a solution of Growth Factor Reduced (GFR) Matrigel:PBS (1:8) at a concentration of 500000 cells/ml and keep them on ice.

Animal Surgery Procedure

I. Use immunocompromised RAG1 -/- mice at 8–12 weeks of age.
II. Before the surgery, inject intraperitoneally the anesthesia solution consisting of Ketamine (65 mg/kg body weight) and Xylacine (13 mg/kg body weight).
III. Place the mice in the warming pad.
IV. Apply Vitamin A ointment (Bausch & Lomb) to the mice, in order to prevent eye dryness.
V. Start the surgery when loss of response to reflex stimulation is observed.
VI. Make an incision about 4 mm long through the skin of the animal to expose the vestibular surface of the hemimandible, along an imaginary line joining the auditory meatus and the lip commissure, to access the muscle layer (**Figure 1A**).
VII. Separate the masseter fibers along their longitudinal axis using a scalpel blade, following an imaginary line parallel to the posterior border of the mouse eye (**Figure 1A**).
VIII. Pay attention not to damage to the blood vessels and keep the muscle retracted using surgical tweezers.
IX. Use a periosteal separator to elevate the periosteum and expose the underlying bone surface.
X. Drill the bone window approximately 2 mm from the posterior border of the ramus, estimating its position using a 1.8 mm dental Woodson condenser (Brassler, Montreal, QC, Canada).
XI. Use a slow-speed dental drill mounting a carbide round burr (Brassler) size 008 to make the bone window (**Figure 1B**).
XII. Irrigate using physiological saline solution during drilling.
XIII. Use a Hamilton syringe Model 702N (with a 22-gauze needle) to inject 10 μl of the prepared solution of DESCs (5000 cells/injection).
XIV. Seal the bone hole using dental canal sealer (AH Plus, Root Canal Sealing Material).
XV. Suture the masseter muscle using absorbable suture 6.0 (Ethicon Inc., Somerville, NJ).
XVI. Suture the skin using non-absorbable silk suture 6.0 (Sherwood Davis & Geck, Wayne, NJ).
XVII. Clean and disinfect the surgical site.
XVIII. Put mice onto a warming pad and observe until they reach consciousness.
XIX. Follow pain management after surgery, by injecting Buprenorphine (0.1 mg/kg bodyweight) subcutaneously, every 6–8 h during the working day and orally administering it overnight, via the drinking water (buprenorphine 0.3 mg/ml are dissolved in 160 ml of water).
XX. Apply Buprenorphine treatment until day three after the surgery.

All mice were maintained and handled according to the Swiss Animal Welfare Law and in compliance with the regulations of the Cantonal Veterinary office, Zurich.

Tissue Processing

I. At the desired time point of analysis, perfuse the mice with freshly prepared 4% Paraformaldehyde (PFA).
II. Dissect the heads and postfix them in 4% PFA overnight at 4°C.

III. Divide the heads in two equal halves along the longitudinal axis.
IV. Dissect the hemimandibles.
V. Decalcify the samples during 6 ± 2 weeks using 10% EDTA at 4°C. Change the EDTA solution every 2–3 days.
VI. Process the samples for paraffin embedding.
VII. Section paraffin blocks at 5 µm and perform immunofluorescence against GFP antibody.
VIII. Analyse the slides with Leica DM6000 FS microscope and take pictures with the Leica DFC350FX camera for the fluorescence imaging and the Leica DFC420C camera for bright-field imaging.

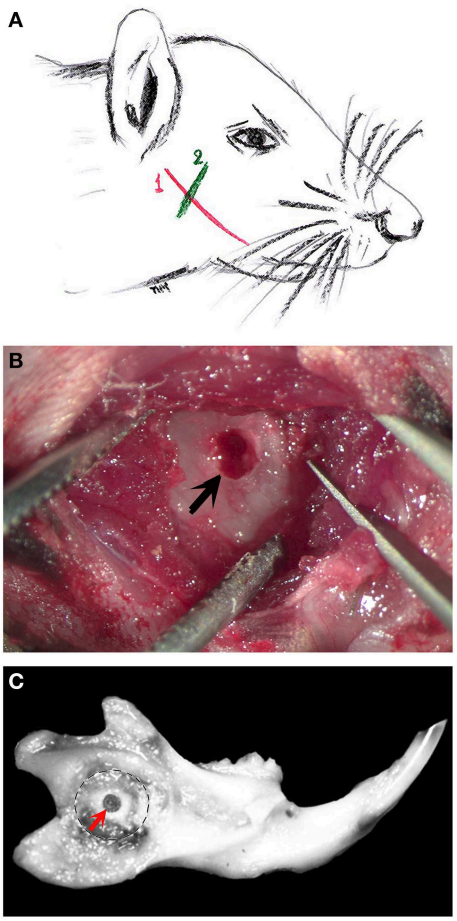

FIGURE 1 | The various steps of the bone "window" technique. (A) Schematic representation of a mouse head showing the incision areas (in red and green colors) in order to expose the alveolar bone of the mouse mandible. The first incision line (in red color, 1) follows an imaginary line that joins the auditory meatus and the lip commissure. The incision is performed through the skin of the animal to expose the masseter muscle. The second incision line (in green color, 2) follows an imaginary line parallel to the posterior border of the mouse eye. This incision serves to separate the masseter muscle fibers in order to expose the alveolar bone in the proximity of the apical end of the incisor. **(B)** After incisions, the drilled bone "window" (arrow) is visible in the exposed mandibular alveolar bone. **(C)** Mouse dissected hemimandible, showing the drilled "window" approximately 2 mm from the incisure of the posterior mandibular border (red arrow).

Results

We have used immunocompromised (RAG1-/-) mice as recipients of DESCs in order to prevent the rejection of the transplanted cells. Thus, all mice recovered well and no complications were observed during the healing period.

The appropriate position of the bone window was confirmed at time of hemimandibles dissection (**Figure 1C**). In all cases the bone windows performed at the labial mandibular bone were drilled very close to the apical part of the incisor. Histologically, there was no alteration of the enamel organ: the drilling did not disrupt the dental tissues and more precisely the external epithelial layer of the incisor (**Figure 2A**). Green fluorescence protein (GFP) positive DESCs were observed in the hole (**Figure 2B**), showing that the GFP-expressing DESCs were successfully delivered to the vicinity of the apical part of the mouse incisor.

Discussion

Cell-based regenerative therapies consist of the *in vivo* administration of stem cells to patients (Mitsiadis et al., 2012). Stem cell transplantation has already been shown to be successful

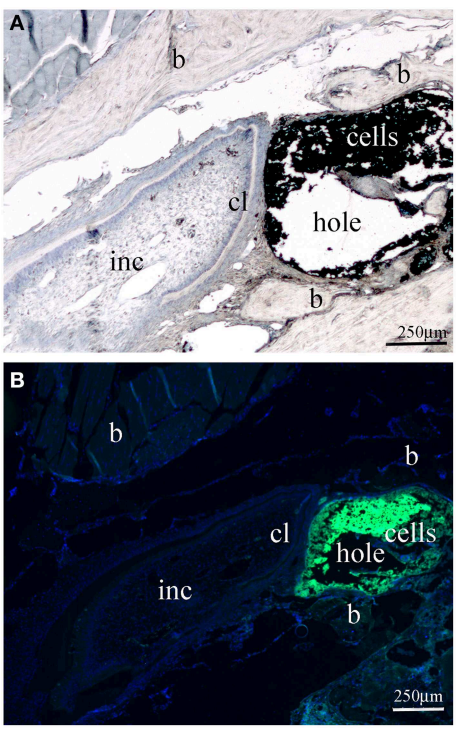

FIGURE 2 | Longitudinal sections through the mouse mandible after the creation of the bone "window" and the injection of GFP-positive dental epithelial stem cells. (A) Light micrograph showing the area of the alveolar bone "window" in close proximity to the posterior end of the mouse incisor. **(B)** Fluorescence micrograph showing the GFP positive dental epithelial stem cells in the drilled hole of the alveolar bone. Abbreviations: b, bone; cl, cervical loop area; inc, incisor. Bars: 250 µm.

for the treatment of several damaged or pathological tissues. For instance, cultured human keratinocyte stem cells have been largely used for the treatment of patients with third-degree burns (Pellegrini et al., 2009). Similarly, human corneal regeneration has been achieved after transplantation of diverse sources of cells such as limbal stem cells (Rama et al., 2010) and oral mucosal epithelial cells (Burillon et al., 2012). Therefore, specific stem cell populations derived from different organs and tissues are extremely interesting for clinical tissue engineering applications. The present step-by-step protocol provides a comprehensive view of a novel experimental procedure for the isolation and local delivery of DESCs in precise areas of the mouse mandible. Isolation of DESCs was based on previous protocols for dental (Chavez et al., 2013, 2014) or other non-dental tissues (Smalley, 2010; De Marval et al., 2014).

Several earlier reports have demonstrated that the formation of a bone window in the rat mandible, where osmotic minipumps can be adapted, constitutes an efficient method for the local and continuous delivery of various substances (Orsini et al., 2001; Nanci et al., 2004), as well as for gene transfer purposes (Wazen et al., 2006). Here we have adapted these techniques in order to develop a new method for *in vivo* stem cells delivery into precise areas of the mouse incisor such as its apical part. This newly described approach would be useful to trace the *in vivo* fate of the DESCs after their injection, and further analyse their integration capacity within the dental tissues.

The bone window technique allows the administration of a relatively high number of stem cells *in situ* that will be necessary for tissue repair and regeneration. However, some caveats cannot be exclused when realizing this technique. For example, because of the confined and narrow space separating the alveolar bone and the underlying dental epithelium, inappropriate position of the hole can either damage the apical end of the incisor or perforate the thin alveolar bone. Another parameter that has to be taken into consideration is time. It is necessary to obtain an efficient strategy for controlling the time period that will eventually vary according to the quantity of injected DESCs. Future developments of this technique are the tracing of injected GFP-positive DESCs and their fate when will incorporate the dental tissues.

To date, this method can be considered a useful *in vivo* approach for delivering DESCs in the mouse incisor. This could lead to greater biologic responsiveness, since the administered cells can endogenously synthesize proteins that may continue to exert its effect *in situ*. Thus, this technique could be easily adapted for the needs of the practitioners in the future. For instance, a potential applications of this technique in humans could be the repair of alveolar bone defects or bone loss during periodontal disease. However, it is still a great challenge to find appropriate sources of cells that ideally could be *in vitro* expanded without losing their regenerative capacity and, in addition, do not cause rejection by the recipient's immune system once transplantated into the target tissue.

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METHODS
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Generation of Spheres from Dental Epithelial Stem Cells

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The *in vitro* three-dimensional sphere model has already been established as an important tool in fundamental sciences. This model facilitates the study of a variety of biological processes including stem cell/niche functions and tissue responses to injury and drugs. Here we describe the complete protocol for the *in vitro* formation of spheres originated from the epithelium of rodent incisors. In addition, we show that in these spheres cell proliferation is maintained, as well as the expression of several key molecules characterizing stem cells such as Sox2 and p63. These epithelial dentospheres could be used as an *in vitro* model system for stem cell research purposes.

Keywords: incisor, tooth, regeneration, epithelial stem cells, mouse, spheres, stem cell niche, 3D culture

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INTRODUCTION

Adult stem cells reside in specific and well-defined areas of most organs and tissues called stem cell niches. Niches provide to stem cells proper signals in order to regulate their function and maintenance according to the requirements of each specific tissue (Li and Xie, 2005; Pagella et al., 2015; Kirkeby et al., 2016). Therefore, the niches contain sources of undifferentiated cells that are involved in both tissue homeostasis and reparative processes after injury (Jiménez-Rojo et al., 2012; Van Keymeulen and Blanpain, 2012). The presence of stem cells is more important in organs with rapid renewal such as hairs, skin and intestine (Blanpain and Fuchs, 2014; Tetteh et al., 2015). The identification of different epithelial stem cell populations with various functions and plasticity during homeostasis and regeneration suggests the complexity of adult epithelial stem cell niches (Blanpain and Fuchs, 2014; Tetteh et al., 2015).

Although monolayer stem cell cultures were established more than three decades ago, three dimensional (3D) *in vitro* systems have recently emerged in order to preserve the physiological microenvironment of the cultured stem cells, thus providing an important tool for both basic and clinical research (Edmondson et al., 2014; Fatehullah et al., 2016; Kretzschmar and Clevers, 2016). Sphere-forming assays have been used to define stemness of adult epithelial cells within organs and tissues (e.g., intestine, mammary glands, and lungs; Dontu et al., 2003). In contrast to the two-dimensional (2D) monolayer culture, 3D culture systems allow cells to grow by forming aggregates/spheroids or organoids (Sasai et al., 2012; Edmondson et al., 2014).

In rodent incisors, dental epithelial stem cells reside in their most posterior part, the cervical loop area (Mitsiadis et al., 2007). The niche formed at the cervical loop is composed by a variety of epithelial cell populations and is separated from the surrounding mesenchyme by a basement membrane (Kieffer-Combeau et al., 2001; Mitsiadis et al., 2007). Hereby, we describe a technique to obtain epithelial dentospheres from the cervical loop of the continuously growing mouse incisor. This method allows evaluating the stemness and plasticity of dental epithelial cells, thus providing with essential information before proceeding with cell-based regenerative approaches in clinics.

MATERIALS AND METHODS

All mice (C57Bl/6) were maintained and handled according to the Swiss Animal Welfare Law and the study was approved by the Cantonal Veterinary office, Zurich (License 11/2014).

Dissection of Cervical Loops from Mouse Incisors (Stereomicroscope)

i. Sacrifice mice by decapitation.
ii. Separate the maxilla and the mandible with a single-edge razor blade.
iii. Separate the two hemimandibles.
iv. Remove the soft tissues around the mandibular alveolar bone.
v. Remove the bone until the incisor is totally exposed.
vi. Press with dissection forceps the bone beneath the cervical loop region to avoid that it remains attached during the next step.
vii. Take the apical part of the incisor with the forceps and pull out the incisor.
viii. Incubate the incisors in Dispase (2 mg/ml) and DNase (20 U/ml) solution in Hank's Balanced Salt Solution (HBSS) for 20 min at RT.
ix. Dissect the posterior area of the incisor that contains the cervical loop area with the help of needles (25G) by separating it mechanically from the mesenchyme.
x. Cut the cervical loop area and add it to a 15 ml falcon tube containing 1 ml of 10% Calf Serum (CS) in Phosphate Buffered Saline (PBS).

Isolation of Single Dental Epithelial Cells (under the Laminar Flow)

xi. Centrifuge the cervical loops in 10% CS/PBS at 300 g for 5 min at RT.
xii. Wash with PBS and centrifuge at 300 g for 5 min at RT.
xiii. Add 250 μl of 0.25% Trypsin (in Leibovitz's L-15 (L-15) Medium) and incubate 20 min at 37°C.
xiv. Pipet up and down.
xv. Add 500 μl DNase I (2 U/ml in L15 medium) and incubate 5 min at 37°C.
xvi. Add 10% CS/PBS.
xvii. Centrifuge at 300 g for 5 min.
xviii. Resuspend the cells in PBS and filter them through a 40 μm cell strainer.
xix. Count the cells.

Embedding of Single Dental Epithelial Stem Cells in Matrigel (under the Laminar Flow)

xx. An aliquot of BD (BD Biosciences) Matrigel™ Basement Membrane Matrix (referred to as Matrigel) should be transferred from −20° to 4°C the afternoon before the day of the experiment.
xxi. Centrifuge the cells at 300 g for 5 min.
xxii. Remove the supernatant and resuspend the cells in the desired volume of culture medium:
- Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12) medium (no phenol red) with B-27 Supplement (1X), Epidermal Growth Factor (EGF) (20 ng/ml), basic Fibroblast Growth Factor (bFGF) (20 ng/ml) and 1% Penicillin/Streptomycin or
- Keratinocyte serum free medium (KSFM) supplemented with EGF and Bovine Pituitary Extract (BPE) and 1% Penicillin/Streptomycin.

For each well (12 well/plate), resuspend 20,000 cells in 50 μl of sphere-forming medium.

xxiii. Add 50 μl of Matrigel to the 50 μl of cells/medium (keeping the tube on ice). Mix gently.
xxiv. Add the 100 μl of Matrigel/cells/medium in the middle of a well (in a 12 well/plate).
xxv. Place the 12 well/plate 1 h at 37°C (in the incubator) to allow the Matrigel to solidify.
xxvi. Add 1 ml of sphere-forming medium to each well.
xxvii. Add 500 μl of fresh medium every second/third day.

Harvesting the Resulting Spheres and Processing Them for Histology

xxviii. Spheres can be harvested 7–10 days after plating the cells.
xxix. Add 500 μl of Dispase II (1 mg/ml) to each well and place the plate 30 mins at 37°C (in the incubator).
xxx. Transfer the volume contained in one well to a 15 ml falcon tube.
xxxi. Centrifuge at 800 g for 5 min.
xxxii. Remove the supernatant and resuspend in 1 ml of 4% PFA. Incubate 20 mins at room temperature.
xxxiii. Centrifuge at 800 g for 5 min.
xxxiv. Remove the supernatant and resuspend in 1 ml of PBS.
xxxv. Centrifuge at 800 g for 5 min.
xxxvi. Remove the supernatant and add the pellet into a plastic cryomold.
xxxvii. Add agarose 1% in the cryomold and let it solidify at room temperature.
xxxviii. Remove the agarose block containing the spheres, place it inside a cassette and process it for paraffin embedding.
xxxix. Section resulting paraffin blocks at 5 μm and perform hematoxylin-eosin staining.
xl. Analyse the slides with Leica DM6000 FS microscope and take pictures with the Leica DFC420C camera.

RESULTS

We have described the method for the dissection of the cervical loop from mouse mandibular incisors and its further dissociation into a single cell suspension (**Figure 1**). We have cultured the isolated epithelial stem cells in a three-dimensional (3D) culture system. Epithelial dentospheres have been obtained by incubating dental epithelial stem cells in a 3D culture system using two different culture media (**Figure 2A**). Thereafter, we have analyzed histologically the dentospheres on 5 μm sections (**Figure 2B**). Interestingly, although the number of

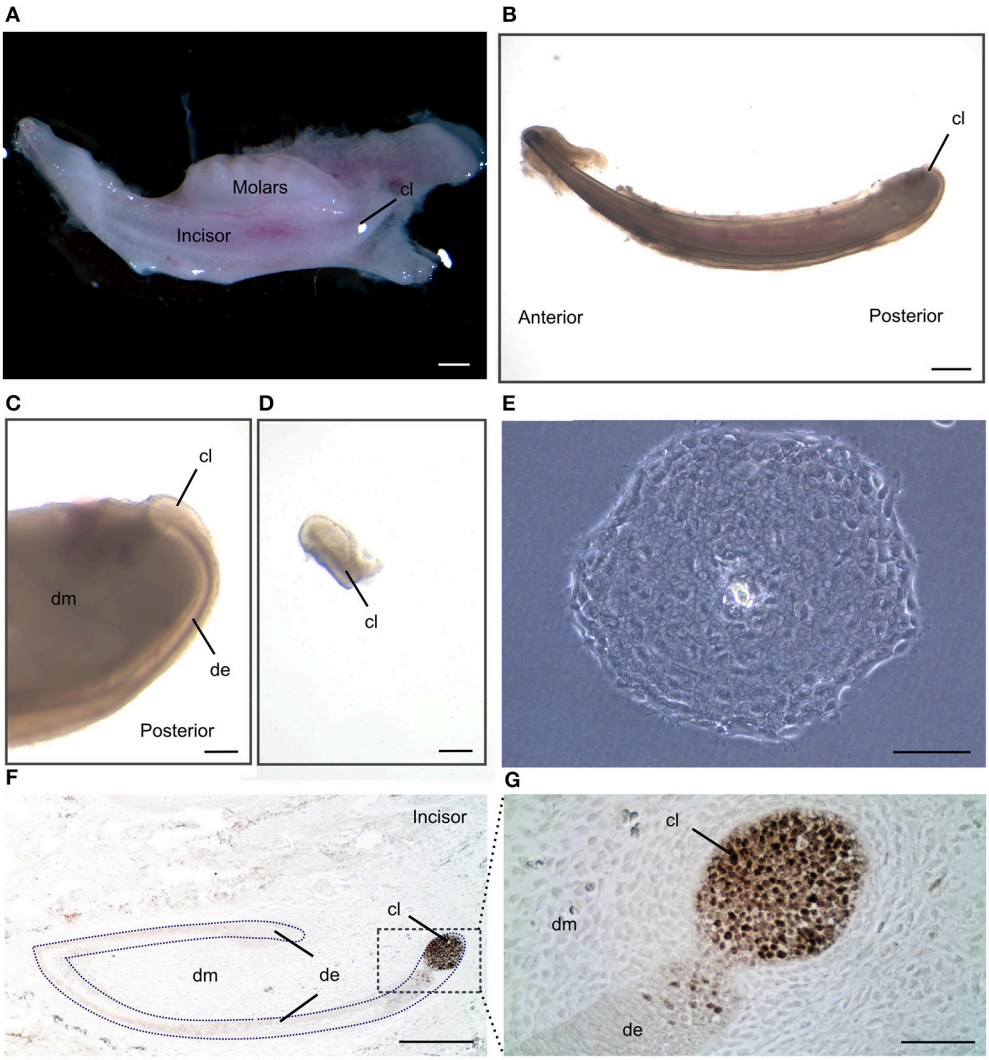

FIGURE 1 | Isolation of dental epithelial stem cells from mouse incisor cervical loop. (A) Mouse mandibular alveolar bone containing incisor and molars. **(B)** Isolated postanal mouse incisor. **(C)** Detail of the posterior part of the incisor where the cervical loop is located. **(D)** Isolated cervical loop. **(E)** Colony derived from a single dental epithelial stem cell from mouse cervical loop cultured in a 2D culture system. **(F)** Immunohistochemistry against the dental epithelial stem cell marker Sox2 and **(G)** a higher magnification of the cervical loop area. Scale bars: 500 μm in **(A,B)**; 100 μm in **(C,D)**; 200 μm in **(E,F)**; 20 μm in **(G)**.

the obtained spheres was not altered when using the two different media (**Figure 2C**), the morphology and size of the spheres (**Figures 2B,D**) was affected and was distinct according to the medium used. More precisely, the spheres formed in presence of Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12) medium containing Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF) differentiated into a stratified squamous-like epithelium with a keratinized center (**Figure 2A**). In contrast, dental epithelial cells cultured in Keratinocyte serum free medium (KSFM) containing EGF and Bovine Pituitary Extract (BPE) formed smaller spheres that lacked any defined organization and not showing a squamous differentiation (**Figures 2A,B**). Histological sections were used to further analyse the protein expression profile of the spheres by both immunohistochemistry and immunofluorescence (**Figure 3**). Staining against the dental epithelial stem cell markers Sox2 and p63 indicate the presence of stem cells inside the epithelial dentospheres obtained after culture in presence of both media. In addition, cells within the spheres proliferate, as it is visualized by nuclear Bromodeoxyuridine (BrdU) incorporation, keep their epithelial identity (indicated by Keratin14 expression), and lack differentiated dental cells (indicated by the absence of Amelogenin). Interestingly, the presence of a set of cells expressing the epidermal terminal differentiation marker Keratin10 was observed within spheres cultured in DMEM/F12 implemented with a cocktail of B27/EGF/bFGF molecules (**Figure 3**). These differentiated cells were less than 15% of the total cell number within the sphere and were situated at the central part of the sphere (**Supplementary Figure 1**).

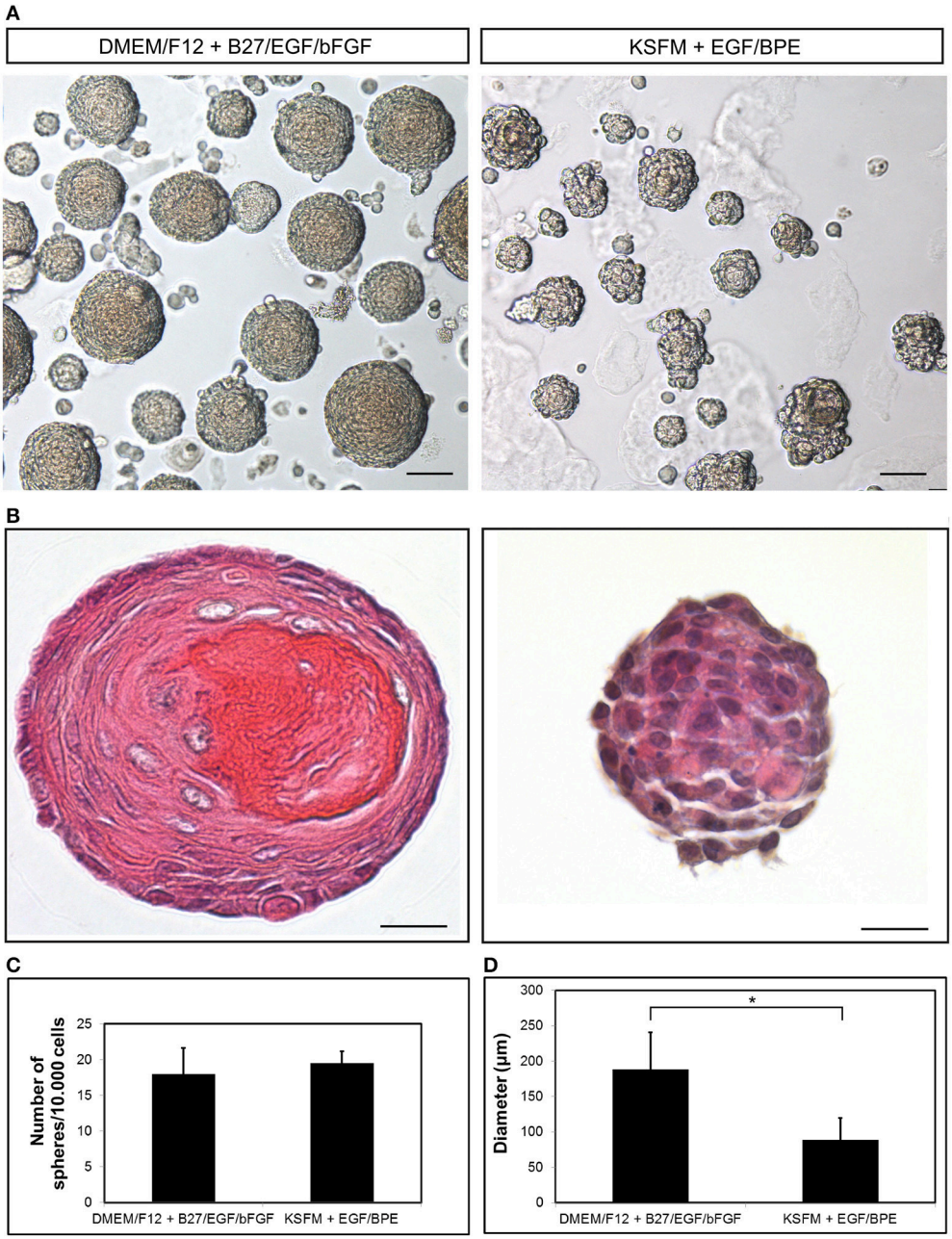

FIGURE 2 | Growth of dental epithelial stem cells as spheres. (A) Epithelium-derived dentospheres were formed in presence of two different culture media. **(B)** Hematoxylin-Eosin staining of the resulting spheres. Graphs showing the **(C)** number and **(D)** diameter of the spheres. Scale bars: 50 μm in **(A)**; 20 μm in **(B)**. *$p < 0.05$.

DISCUSSION

Here we describe in detail the various steps leading to the generation of epithelial dentospheres, which were then analyzed histologically and molecularly. Sphere-forming assays were firstly used in the neural stem cell field, where neurospheres were generated from cells of the adult central nervous system (Reynolds and Weiss, 1992). Afterwards, these assays have been largely used to study the behavior and stemness of putative stem cells (Dontu et al., 2003; Yoshida et al., 2005; Edmondson et al., 2014). In teeth, spheres (or dentospheres) have been generated mostly from their mesenchymal component such as the dental pulp and the dental follicle (Miura et al., 2003; Sasaki et al., 2008; Stevens et al., 2008; Abe et al., 2011; Keeve et al., 2013). The formation of spheres originated from dental epithelial tissues retained less attention and therefore remains

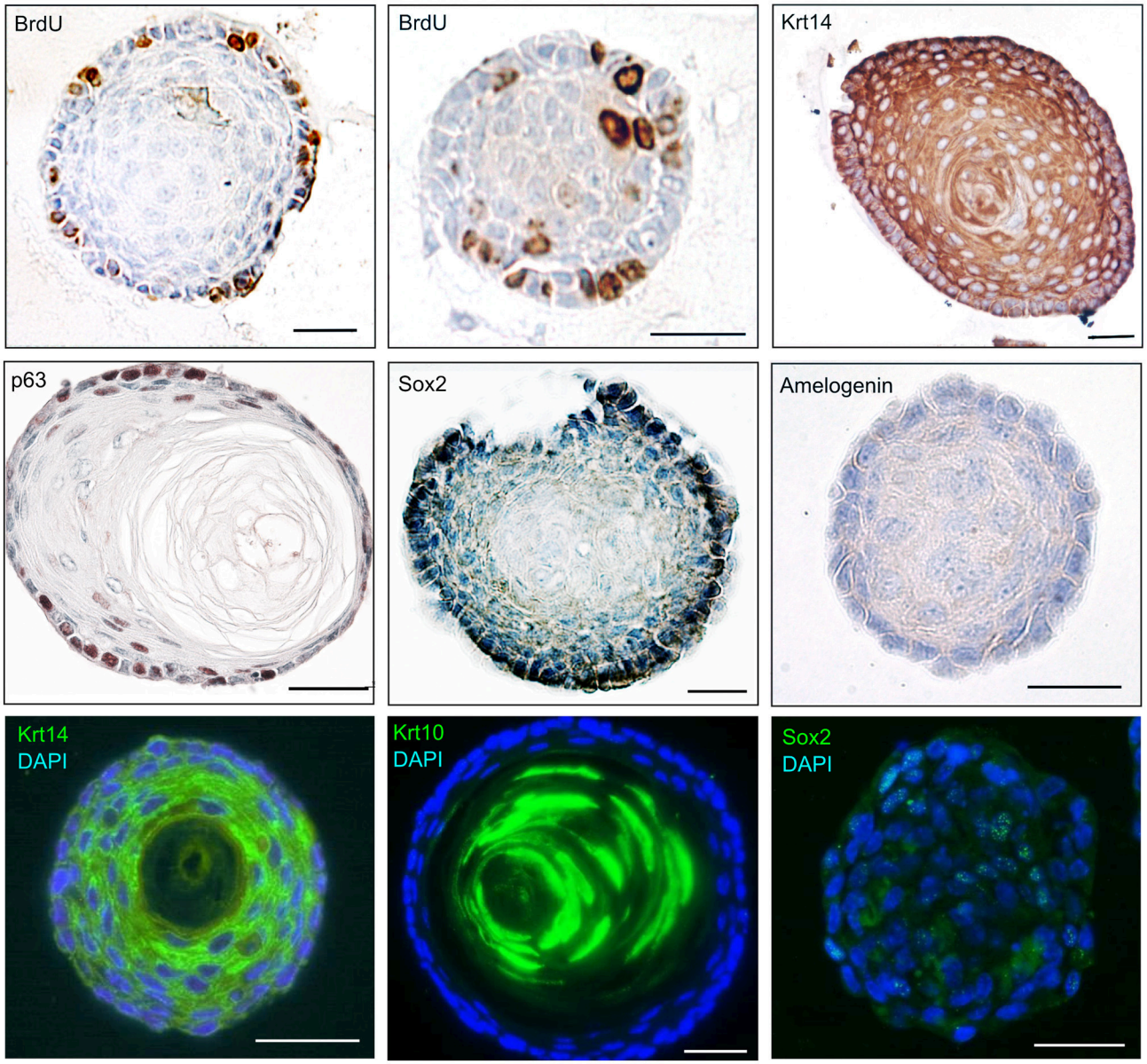

FIGURE 3 | Molecular profile of epithelial dentospheres. Immunohistochemistry and immunofluorescence on epithelial dentospheres showing BrdU incorporation in proliferating cells, and expression of specific markers showing the epithelial identity (Krt10, Krt14) and stemness (p63, Sox2) of cells. Absence of Amelogenin indicates that cells within dentospheres are not differentiated into dental specific epithelial cells (ameloblasts). Abbreviations: BrdU, Bromodeoxyuridine; Krt10, Keratin10; Krt14, Keratin14. Scale bars: 30 μm.

poorly unexplored. The few realized studies have shown that dental epithelial cells isolated from the cervical loop area of postnatal mouse incisors are able to form spheres in serum-free 3D culture systems (Chang et al., 2013a,b; Chavez et al., 2013, 2014). Interestingly, the resulting spheres presented varying sizes and morphogies depending on the composition of the culture media used in the assays. For instance, when using a medium similar to that used previously for generating neurospheres, which consists of Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F12) medium containing EGF and bFGF, dental epithelial stem cells formed well-delimited round-shaped spheres (Chavez et al., 2013, 2014). However, dental epithelial stem cells cultured in an epithelial-specific medium formed spheres that are smaller in size when compared to those obtained with the above mentioned medium (Chang et al., 2013a,b).

Here we show that appropriate selection of signaling molecules is important in order to allow maintenance and proliferation of stem cells within the spheres. Indeed, cells cultured in presence of DMEM/F12 with B27/EGF/bFGF generated spheres with multiple layers, which closely resemble to keratinized stratified squamous epithelial structures. In contrast, in presence of a medium composed by KSFM with

EGF/BPE, the spheres adopted a more irregular morphology, where layers were not clearly distiguisable in histological sections. Immunohistochemistry and immunofluorecence revealed that spheres generated in both culture conditions contain cells that proliferate, as shown by BrdU labeling and, furthermore, express the stem cell markers Sox2 and p63. Spheres formed in presence of DMEM/F12 supplemented with B27/EGF/bFGF molecules exhibited a regionalization with clear undifferentiated and differentiated territories, both histologically and molecularly. Indeed, proliferative events and expression of specific stem cell markers were evident in the most external layers of the sphere, thus resembling to the basal layer of keratinized epithelial structures where stem cells reside. In the same spheres, differentiated epithelial cells located in the center could be considered as equivalent to cells of the suprabasal layers of a keratinized epithelium. In contrast, in spheres formed in presence of KSFM with EGF/BPE all cells exhibited a uniform morphology and expressed the Sox2 stem cell marker, but not a terminal differentiation marker, thus suggesting that these spheres contain only cells with stemness.

These results strongly suggest that the epithelial stemness has been retained in the spheres generated by both protocols used. This is reinforced by the capacity of cells originated by the spheres to *de novo* recreate spheres (secondary spheres) when placed in identical culture conditions.

In summary, the methods described here are valuable to assess the stemness of dental epithelial cells, making epithelial dentospheres one promising model for studying tooth pathology and regeneration.

AUTHOR CONTRIBUTIONS

DN, ZG, and LJ performed the experiments. All authors contributed equally to the experimentation plan, writing and analyzing results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00007/full#supplementary-material

Supplementary Figure 1 | Percentage of undifferentiated vs. differentiated cells within the spheres. Keratin10 positive terminally differentiated cells constitute less than 15% of the total cell number within the epithelial dentospheres.

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